Relevance of Humoral Immunity in Neuro-inflammatory Disease

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List of publications included in this thesis:

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Autoantibody-mediated demyelination depends on complement activation but not activatory Fc-receptors

Proc. Natl. Acad. Sci. USA 103, 18697-18702 (2006)

E. Urich, L.M. Howard, S.D. Miller, A. Waisman and B. Becher

Importance of B cells as APCs in neurodegenerative diseases

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E. Urich and B. Becher

Role of B cells and humoral Immunity in Multiple Sclerosis

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Abbreviations

Abs	antibodies
Ag	antigen
APC	antigen presenting cell
Auto-Abs	autoreactive antibodies
BBB	blood brain barrier
BCR	B cell receptor
BM	bone marrow
bp	base pair
CFA	complete freund`s adjuvant
CFSE	carboxy fluorescein diacetat succinimide ester
CNS	central nervous system
CSF	cerebrospinal fluid
Con A	concanavilin A
CPM	counts per minute
CCP	classical complement pathway
CR	complement receptor
DC	dendritic cell
DPI	days post immunization
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FcγR	Fcγ receptor
$FcR\gamma^{-/-}$ mice	FcRγ-chain knockout mice

FDC	follicular dendritic cell
FITC	fluorescein-isothiocyanate
GM-CSF	granulocyte macrophage colony-stimulating factor
H&E	haematoxylin & eosin
IC	immune complexes
Ig	immunoglobulins
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
ITAM	intracellular immunoreceptor tyrosine-based activation motif
ITIM	intracellular immunoreceptor tyrosine-based inhibitory motif
LFB	luxol fast blue
KLH	keyhole limpet hemocyanin
KO	knock out
LN	lymph node(s)
LPS	lipopolysaccharide
LTβR-Fc	lymphotoxin beta receptor Fc-fusion protein
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NK-cells	natural killer-cells
OCB	oligoclonal immunoglobulin bands
OD	optical density

ODC	oligodendrocyte(s)
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll-a protein
PLP	proteolipid proteins
PTX	pertussis toxin
rMOG	recombinant MOG, derived from rat (rrMOG), mouse (rmMOG) or human (rhMOG) sequence
RRMS	relapsing remitting MS
s.c.	subcutaneous
TCR	T cell recetpor
Tg	transgen(ic)
TGF	tumor growth factor
T_H	T-helper cell
TNF	tumor necrosis factor
WT mice	wild-type mice

Outline of the thesis:

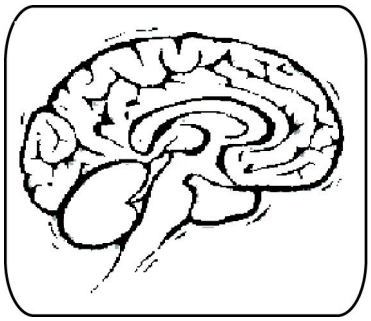
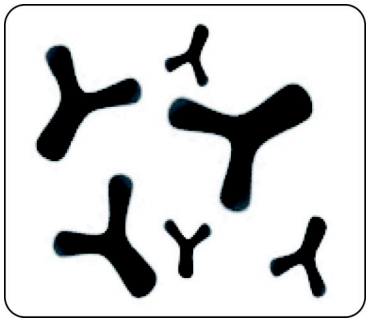
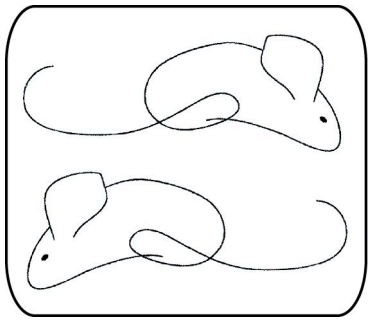
The precise role of B cells and humoral immunity in both Multiple Sclerosis and its animal model EAE remains a subject of intense debate. While intrathecal immunoglobulin (Ig) production and Ig-deposition in inflammatory lesions is a hallmark of MS, mice deficient in B cells and Igs have been shown to develop severe EAE. Paradoxically, mice deficient in FcRγ are resistant to EAE-induction. We found that the functional expression of FcRγ on systemic accessory cells, but not CNS-resident cells, appears to be vital for the development of CNS-inflammation, independent of APC function or antibody (Abs) involvement. While we can clearly dismiss B cells as mediators in actively, MOG-peptide induced EAE, we found that the injection of auto-Abs directed against MOG drastically worsens disease severity, inflammation and demyelination. The action of such Abs is again FcR-independent and relies entirely on complement activation, as they have no detrimental effect in $C1q^{-/-}$ mice. We conclude that Abs generated during the course of EAE induced by active immunization are irrelevant for the pathogenesis of EAE, but that under certain conditions Abs are capable of driving demyelination in an FcRγ-independent but complement-dependent fashion. Anti-myelin Abs are thought to contribute to MS pathogenesis in a subpopulation of patients, possibly through crosslinking of FcγR and complement activation, although the relative contribution of these effector pathways is unclear and requires further study. The studies described in this thesis aimed to further elucidate the relevance of B cells as APCs in EAE pathogenesis. The inability of MOG protein to induce EAE in B cell-deficient mice indicate that B cells are of key importance for the induction of EAE by a large polypeptide, even though our data provide evidence that B cells are neglectable as APCs, for protein as well as for peptide Ag.

Zusammenfassung:

Die Krankheitsmechanismen der MS sind größtenteils unverstanden und die wichtigsten Erkenntnisgewinne beziehen sich auf die experimentelle Forschung am klassischen Tiermodell für die MS, der EAE. Während sich bei MS die Hinweise einer Beteiligung von B-Zellen und Immunglobulinen (Ig) häufen, konnten voneinander unabhängige Forschungsgruppen zeigen das B–Zell-defiziente Mäuse genauso anfällig sind für die MOG-Peptid vermittelte EAE wie ihre Wildtyp-Kontrollen. Es konnte aber auch gezeigt werden, dass Myelinprotein-spezifische Antikörper in der Lage sind die Demyelinsierung zu verstärken und die Effektorfunktionen während der EAE voranzutreiben. Die Möglichkeit das B Zellen aufgrund ihrer Fähigkeit Antigene (Ag) zu präsentieren die EAE-Pathogenese beeinflussen könnten, wurde mit dieser Arbeit widerlegt. Nicht zuletzt deshalb, weil wir zeigen konnten dass die EAE Resistenz B–Zell-defizienter Mäuse bei Immunisierung mit MOG Protein anstelle von kurzkettigem Peptide, keinen Einfluss auf die Proliferation krankheitsverursachender T Zellen hat. Im Gegensatz zu all diesen Befunden sind Fc-Rezeptor-defiziente Mäuse ($FcR\gamma^{-/-}$) durchweg resistent gegenüber der EAE-Induktion oder zeigen erheblich mildere Verlaufsformen der Krankheit. Dies steht im offensichtlichen Widerspruch zu den mit den B-Zell-defizienten Mäusen gemachten Beobachtungen. Wir fanden heraus, dass die beobachtete EAE Resistenz der $FcR\gamma^{-/-}$ Mäusen komplett unabhängig von einer Ig-Bindung und deren Folgen ist und das tatsächlich eine alternative Funktion der FcRγ Kette, auf einem nicht im ZNZ angesiedelten Leukozyten, existiert. Desweiteren war es uns möglich zu zeigen, dass die krankheitsverstärkenden Eigenschaften autoreaktiver anti-MOG Antikörper nicht FcR mediert sind, sondern vielmehr deren Effektorfunktionen durch das klassische Komplementsystem vermittelt werden.

Relevance of humoral immunity

in neuro-inflammatory disease

Chapter 1

General Introduction

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1. Multiple Sclerosis

MS is an inflammatory disease that affects the central nervous system (CNS), i.e. the brain and spinal cord, and usually starts between 20 and 40 years of age. Clinical signs are heterogeneous, including a wide variety of motor deficits such as muscle weakness, tremor and paralysis, often accompanied by sensory deficits, such as visual impairment[1]. The prevalence of MS varies significantly depending on the genetic background of the patient[2]. A considerable number of genetic polymorphisms, mostly in genes related to the immune system, have been associated with MS susceptibility[3]. Even in areas with high prevalence, MS is more prevalent in Caucasians and only rarely observed in Asians or Africans. Moreover, the risk of developing the disease is significantly higher in family members of patients with MS[4]. Environmental factors implicated in MS pathogenesis, include geographical differences in exposure to sunlight (vitamin D) and infections[5]. The disease process in MS is characterized by large, sharply demarcated areas of demyelination in the CNS, associated with macrophage and B cell infiltration, accumulation of perivascular and parenchymal T cells, and activated microglia. Tissue damage, including loss of neurons and oligodendrocytes (ODCs), astrogliosis and remyelination, accompanies the inflammatory changes[6].

2. EAE

Immunological processes contributing to CNS inflammation and demyelination, including the role of humoral immunity, are mostly studied in animal model of MS, the EAE (experimental autoimmune encephalomyelitis). EAE shares many features with MS, such as acute, chronic and relapsing neurological

dysfunction, paralysis and ataxia, and strikingly similar histopathological changes depending on the model[7]. EAE can be induced in a number of susceptible animals by immunisation with CNS homogenate or purified myelin proteins, such as MOG (myelin oligodendrocyte glycoprotein), PLP (proteolipid protein) or MBP (myelin basic protein) emulsified in complete Freund`s adjuvant (CFA) (active immunisation)[8]. Generally, immunisation with myelin antigens induces a T cell mediated autoimmune response. T cells specific for myelin antigens are activated in the periphery, migrate to the CNS, cross the blood brain barrier (BBB) and enter the CNS parenchyma. Local re-activation of myelin specific T cells results in the activation of microglia and perivascular macrophages and the recruitment of peripheral blood immune cells, to the CNS parenchyma[9]. Invading immune cells, such as macrophages, B cells, NK cells, granulocytes and $CD8^+$ cells then start a local inflammatory and demyelinating response that results in the motor deficits, which are the read-out system for clinical EAE. The role of an autoimmune response and the pivotal importance of T cells in this model was proven by adoptive transfer experiments, which showed that activated $CD4^+$ T cells from a diseased animal can transmit disease to naïve animals[10]. However, the characteristics of EAE in the different models, such as disease incidence, day of disease onset, disease severity and the extent of CNS inflammation and demyelination may depend on additional factors, such as the activation of the humoral immune response[6].

3. Humoral Immunity in MS and EAE

The contribution of the B cells, Abs and complement in EAE is highly variable in between the different models, and therefore it is often difficult to compare the

results obtained in the different studies. This paragraph, will review the studies of humoral immunity mainly in EAE but also in MS and interpret the results. Furthermore it will address the relevance of FcγR and complement in Ab-mediated demyelination.

3.1. B cells and antibodies

Although B cells are known to be associated with MS lesions, where they can potentially contribute to lesion formation by the production of myelin specific Abs[11-16], information on their physiological and pathological function is still sparse. An increased intrathecal production of Immunoglobulins (Igs) in the cerebrospinal fluid (CSF) is evident in more than 90% of MS patients[17, 18]. It was found that the increased Ig concentration in CSF of MS patients correlates with episodes of MS relapses, and MS patients lacking oligoclonal bands (OCB) are considered to develop a more benign disease course[19]. Generally, the reperoire of anti-myelin Abs in the serum is more diverse and less specific for MS than those in the CSF. The antigen (Ag) specificity of OCBs in MS patients however is largely unknown. It has been reported that OCBs are detected against MBP and viral Ags, although generally the IgGs in the OCBs do not represent responses against myelin or infectious agents[20]. It is possible that OCBs in MS are directed against Ags that are as yet unknown (i.e. viral Ags or modified self Ags). OCBs are not only detected in CSF, but can also be eluted from MS lesions[21], suggesting that at least part of the OCBs found in the CSF are produced at the site of demyelination. In addition, anti-myelin Ab responses may vary in different stages of the disease as a result of spreading of B cell epitopes, as has been described for T cell epitopes in MS[22] and as is repeatedly

described in EAE[23]. Beside the notion that B cells can contribute to autoimmune disease directly, or indirectly via forming immune complexes (ICs)[24-26] or as regulatory cells by the production of cytokines (IL-10, TGF-β)[27, 28]. B cells however also have the capacity to promote T cell activation[29-32], as antigen presenting cells (APCs) for priming naïve $CD4^{+}$[33-35] or memory $CD4^{+}$ T cells[36], although B cells seems to be limited in their ability to function by acting as primary APCs[37]. Furthermore Ag presentation to T cells by B cells often seems to be unfavorable for cellular responses and lowers the amount of Ag available for other APCs.

The precise role of B cells and the relevance of anti-myelin Abs in EAE is a subject of intense debate[27, 28, 38-46]. In EAE, no direct correlation between neuroantigen-specific Ig titers and disease severity could be demonstrated[38]. EAE studies in mice genetically deficient in B cells (μMT) using peptide of MOG and MBP found no requirement for B cells or Abs in disease induction or demyelination[39-43]. On the contrary, B cells are found to exert regulatory effects and IL-10 producing B cells are thought to limit the extent of immunity against the CNS[27, 28]. Others however claim that B cells and Abs are, in general or under certain conditions, important for the disease process[40, 41, 47-51]. Transgenic expression of a B cell receptor specific for MOG leads to an aggravated form of disease after immunization and anti-myelin Abs exacerbate CNS inflammation and demyelination in EAE[44]. Although μMT mice develop EAE following immunization with an encephalitogenic MOG peptide (MOG_{35-55}), they were found to be resistant to disease induction via immunization with human but not rat recombinant MOG protein[40, 47, 48]. Rat rMOG protein, which contains the

encephalitogenic rodent peptide, induce EAE in the absence of B cells but not human rMOG, although human MOG_{35-55} is encephalitogenic[48]. These findings indicate that protein-induced EAE is mediated in part by B cells acting as APCs and that a single amino acid substitution in human rMOG (position 42), changes the mechanism of encephalitogenicity from B cell dependent to independent[45-47]. However, this finding was disputed by Fillatreau et al.[27].

Box 1.1. B cells and Immunoglobulins

B cells originate from lymphoid precursors in the bone marrow and account for 10-15% of blood lymphocytes. Their main function are production of immunoglobulins (Ig) and antigen presentation to T cells. Antigen (Ag) specificity of B cells is determined by the B cell receptor (BCR) that consists of a membrane bound Ig molecule associated with the $Ig\alpha Ig\beta$ heterodimer. Mature naïve B cells express low levels of low affinity membrane bound IgM molecules. Encounter of specific Ag induces B cell activation and differentiation, resulting in secretion of low affinity IgM in pentameric form. B cell activation occurs predominantly in the lymph nodes (LN), where follicular dendritic cells (FDCs), that have captured Ag in the form of immune complexes (ICs) present Ag to B cells. Like T cells, B cells require more than just the presence of Ag to become fully activated. The additional activating signal can be provided by $CD4^+$ T cells or by microbial Ag.

T cell-dependent B cell activation. After binding to protein Ag, B cells internalise the BCR-Ag complex, process the Ag and present it as a peptide on MHCII molecules. Recognition of the MHC-peptide complex by $CD4^+$ T cells results in activation of B cells, but also in enhanced activation of the T cells through costimulatory interactions (such as CD40-CD40L), and B7-CD28). B cells subsequently undergo somatic hypermutation of the IgV genes, resulting in the selection of high affinity BCR and production of high affinity antibodies (Abs) (affinity maturation). In addition, rearrangement of the heavy chain results in the selection of another IgFc tail, changing the Abs isotype from IgM to IgG, IgA or IgE (isotype switching), alters antibody effector function without altering Ag specificity. Differentiation and affinity maturation results in the generation of memory B cells that express a high affinity BCR, produce low levels of Abs and can undergo new cycles of activation and differentiation upon reencounter with the Ag. Alternatively, B cells may develop into end-differentiated plasma cells that have lost all surface Igs and continuously secrete high levels of Abs.

T cell-independent B cell activation. High doses of microbial Ag activate B cells regardless of Ag specificity (polyclonal activation), whereas lower doses only activate Ag specific B cells although without T cell help. The alternative route of T cell-independent B cell activation is by repeating linear Ag that simultaneously crosslink multiple Ag specific BCR. Isotype switching or development of memory B cells does not occur after T cell independent B cell activation.

Antibodies and Immune complexes. Immunoglobulins (Igs) bind Ag in their native form, including conformational epitopes. This is in contrast to T cells, which recognize peptide Ags (linear epitopes) in the context of MHC molecules. Binding of Abs to soluble Ag results in the formation of immune complexes (IC), whereas binding to particulate Ag, expressed on the surface of microorganisms or cells, results in opsonisation of the particle, efficiently targeting the particles removal by mononuclear phagocytes. The effector functions of Igs depend on the Ig isotype. Binding of pentameric IgM to Ag results in efficient complement fixation, facilitating capture and removal of Ag by phagocytes. IgG-containing IC are capable of complement fixation and crosslinking of leukocyte Fcγ receptors (FcγR), including a variety of effector function (including FcγR mediated phagocytosis, Ag presentation and Abs dependent cytotoxicity). IgA Abs are secreted as dimers in the lumina of mucosal surfaces and mainly act as neutralising Abs, in blood IgA occurs in monomers with unknown function. IgE Abs acts as Ag receptors on mast cells by Fc-mediated binding to mast cell Fcε receptors that are capable of inducing potent inflammatory reactions after cross-linking by Ag[52].

B cells as antigen presenting cells (APC). Although B cells are poor activators of naïve T cells, memory B cells are potential APCs that were shown to be important for propagation of T cell responses in several autoimmune disease, including classical T cell mediated diseases[53]

3.2. Fc-receptors

Whereas the role of Igs in MS and EAE remains enigmatic, the function of Ig-binding molecules (Fc-receptors) in neuroinflammation is equally controversial. Several studies characterized the presence and expression Fcγ receptors (FcγR) in MS. All three subclasses of FcγRs (FcγRI, FcγRII and FcγRIII), are constituively expressed by ramified microglia and perivascular macrophages in the CNS[54]. FcγR expression is enhanced on lipid-laden macrophages in active MS lesions[55]. Prineas and Graham[56], showed capping of phagocytic macrophages with IgG in MS lesions, suggestive of Ab mediated phagocytosis. However, colocalisation of FcγR with IgG has not been described in MS lesions, the binding of auto-Abs to myelin particles may trigger the inflammatory process by activation of FcγR on microglia and macrophages in the CNS[57-59]. The capacity of FcγR to contribute to myelin phagocytosis was demonstrated *in vitro*, where myelin specific Abs enhanced myelin phagocytosis in the absence of active complement[60]. In addition, cultured microglia produced inflammatory chemokines upon FcγR crosslinking, supporting a pathogenic role for IgG-FcγR interactions in the CNS[61]. Genetic differences between FcRs have been described in patients with autoimmune diseases[62-66]. Myhr et al.[62] reported an association between FcγR genotypes and MS disease course in 136 Norwegian MS patients. Nevertheless because FcγR genes are in close proximity to each other on human chormosome 1, it remains possible that they are in linkage disequilibrium with each other, so correlations with one FcγR genotype might in fact implicate other FcγR genes in disease susceptibility. The balance between activatory and inhibitory FcγR was shown to be very important in Ab-mediated autoimmune disease. FcRγ chain knockout mice ($FcR\gamma^{-/-}$), lacking the activatory FcγRI and FcγRIII[67], were protected from collagen- and immune complex-induced arthritis[68], glomerulonephritis[69], and vaculitis[70]. In contrast, mice deficient in the inhibitory FcγRII developed spontaneous glomerulonephritis[71] and were more sensitive to collagen-induced arthritis[72].

It is also well documented that the FcR γ-chain (FcRγ) is important in the development of EAE[73]. $FcR\gamma^{-/-}$ mice are EAE resistant[74-76], even though they develope comparable levels of MOG-specific Abs[74, 75]. By contrast, the lack of

inhibitory FcγRIIb enhanced susceptibility to MOG-induced EAE and increased the extent of demyelination[75]. This led to the conclusion that interactions between anti-myelin Abs and FcγR are important for the induction of EAE and that the resistance of FcRγ$^{-/-}$ mice to EAE is due to the inefficient antigen processing or presentation of myelin proteins during the induction of secondary immune response locally in the CNS. FcRγ is not only critical for FcR-mediated signaling, but this ITAM motif-containing γ-chain is further involved in the signal transduction of several other FcR-unrelated receptor complexes on different cell types. These include NKR-P1 on NK-, NK T- and dendritic-cells (DCs); Pir-A on DCs, macrophages, B cells and platelets. Furthermore FcRγ is closely related to the ζ-chain of the T cell antigen receptor (TCR)/CD3 complex and in fact FcRγ-ζ heterodimers and FcRγ-FcRγ homodimers can participate in TCR formation and function[77-84]. Indeed, Szalai et al. have even suggested that FcRγ signaling on γ/δ T cells could explain the resistance of FcRγ$^{-/-}$ mice to EAE[77]. They implied that expression of FcRγ by γδ T cells, probably in conjunction with the T cell receptor/CD3 complex, is potentially required for full development of EAE.

Box 1.2. Fc receptors (FcR)

Fc-receptors (FcR) function as a bridge between humoral and cellular immunity by orchestrating the interaction of Abs with effector cells. Two general groups of FcR can be distinguished: those expressed by leukocytes that are specific for each of the five immunoglbulins classes (IgA, IgE, IgG, IgM and IgD) and that trigger effector functions, and those that mediate transport of Igs across epithelial or endothelial surfaces (poly-Ig receptor and FcRn)[85].

Fcγ receptors (FcγR), play a critical role in immunity by linking IgG Ab mediated responses with cellular effector and regulatory functions of the immune system[86].

Human FcγR consists of three FcγR subclasses, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), all of which are encoded by genes on chromosome 1. FcγR belongs to the Ig superfamily and contain two (FcγRII and III) or three (FcγRI) Ig-like extracellular domains, one transmembrane domain and cytoplasmic domains of variable length[65]. For optimal surface expression and signal transduction FcγRI depends on accociation with a dimer of the FcRγ chain that contains an intracellular immunoreceptor tyrosine-based activation motif (ITAM). In the absence of the FcRγ chain, surface expression of FcγRI is severely reduced and most effector functions are severely impaired. Human leukocytes express two functional FcγRII, FcγRIIa and FcγRIIb. FcγRIIa is an activating receptor that contains an intracellular ITAM, and is independent of the FcRγ chain for functional expression and signalling. FcγRIIb is the only inhibitory FcγR found in humans and has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its intracellular domain. FcγRIIIa is a transmembrane receptor that is dependent on association with a signalling molecule for functional expression. FcγRIIIa primarily associates with a dimer of FcRγ chains, but association with a dimer of CD3ζ chains or a heterodimer of the γ and ζ chains is also possible[87]. FcγRIIIb is a glycophosphatidylinositol (GPI) linked molecule that lacks a transmembrane domain. FcγRIIIb is thought to capture IC without inducing inflammation[88].

Murine FcγR Several differences exist beween the human and murine FcγR system. Murine FcγRI is encoded on chromosome 3 whereas FcγRII and FcγRIII are encoded on chromosome 1. Murine FcγRI depends on FcRγ chain for signal transduction and surface expression[67]. Murine FcγRIIIa is also fully dependent on a dimer of FcRγ chain for surface expression and signalling.

3.3. Classical complement system

The complement system is well represented in the CNS. Glia cells and neurons express all of the activation and regulatory proteins and the C3a/C5a receptors[89-92]. It has been suggested that the activation of the complement system is involved in the pathogenesis of several neurodegenerative disease including Alzheimer`s[93, 94], Parkinson`s, Huntington`s, Prion diseases[95, 96], and MS[58, 97-103]. Enhanced expression of mRNA for C1q and to a lesser extent C3, in MS lesions demonstrates that at least part of the complement proteins are produced locally in areas of active demyelination[104, 105]. Gene-microarray analysis of active MS lesions also previously showed enhanced mRNA expression for C1r[76], a protein that forms the C1 complex with C1q and C1s. In a subgroup of early MS patients, deposition of IgG, anti-myelin Abs and

complement was detected together in active demyelination lesions in situ[11, 15, 58, 106, 107]. This suggests that Ab-mediated activation of complement is involved in the demyelinating process in a subpopulation of MS patients.

The role of complement activation in EAE has been hotly debated over the past years[108-110]. Loss of C3 apparently attenuates EAE[111] and expression of the complement inhibitor sCrry prevents EAE[112]. On the one hand complement activation appears to be crucial in the context of EAE exacerbated by the inoculation of demyelinating Abs[57, 113-115], and on the other hand, studies[109, 110] could demonstrate that key components of the complement system, such as C3 and C4, are not involved in MOG_{35-55} induced EAE pathogenesis challenging the notion that complement activation drives demyelination. The capacity of anti-myelin Abs to exacerbate clinical signs of EAE is mostly restricted to Abs directed against MOG and indeed the Abs that could fix complement most efficiently had the highest demyelinating potential[113]. Previous studies demonstrated that complement depletion cannot[116], or not completely[117], prevent acute exacerbation of EAE by anti-MOG Abs implicating other effector mechanisms such as ADCC. While these findings are indicative of a role of the complement system, they provide by no means conclusive evidence. Our study of anti-MOG Ab-exacerbated EAE in complement deficient mice, definitively implicates the complement system as the dominant if not the only effector cascade invoked by demyelinating Abs[118].

Box 1.3. The complement system

The complement system is composed of a large number of serum proteins and membrane-bound receptors that serve to protect the host from pathogens through innate and adaptive immune mechanisms[119, 120]. Activation of the complement system is initiated by binding of complement to immune complexes (IC) (classical complement system), pathogens (alternative pathway) or modified self-antigens. Binding of the first complement component induces a cascade of reactions that results in opsonisation or lysis of pathogens. Complement components are also involved in clearance of apoptotic cells, inflammation and tissues destruction.

The classical pathway is activated by binding of C1q to IC or apoptotic cells[121]. C1q is the first subcomponent of the C1 complex and initiates the complement cascade`s classical pathway by binding the FcR-protion of IgG engaged in ICs[122]. In its inactive state, C1q forms a complex with C1r and C1s. This complex disintegrates upon binding of C1q to IC, releasing C1r and C1s from the complex, thereby exposing an enzymatic site on C1r that cleaves C1s to become an active protease. C1s then initiates a cascade of reactions, leading to cleavage of complement C4, C2, C3 and C5. After cleavage one part of the protein is released as an inflammatory mediator (C2a, C4a, C3a and C5a) and the other part either acts as a new enzyme (C2b) to cleave other complement components or binds to the pathogenic or apoptotic surface as an opsonin (C4b, C3b). Binding of C5b to C3b on the opsonised surface initiates the final pathway of complement that is shared by all three pathways. C5b forms complexes with C6, C7 and C8, which insert itself into the membrane. C9 molecules then bind to this complex and polymerise, forming a pore in the pathogenic membrane – the membrane attack complex (MAC) – that contributes to lysis of the pathogens[120].

The Mannan-binding lectin (MBL) pathway is initiated by binding of mannose binding lectin to microbial pathogens[123], ficolins[124], and IgA-containing immune complexes[125], followed by cleaving of C4 and C2 and further activation of the complement cascade similarly to the classical pathway.

The alternative pathway is activated by direct binding of C3 to pathogens, followed by activation of series of proteins (factor B, factor D and factor P) that amplify the response by cleaving more C3 into C3a and C3b. C5b then binds to C3b, initiating the final pathway of complement.

Complement receptors. After complement opsonisation, pathogens or apoptotic cells can induce leukocyte activation or phagocytosis mediated by complement receptor (CR)[126]. Mouse CR1 (CD35) and CR2 (CD21) are alternatively spliced products of the same Cr2 gene, while in humans these proteins are derived from two distinct but closely linked genes on chromosome 1[127]. CD35/CD21 is expressed primarily by mature B cells and follicular dendritic cells (FDC) where it associates with CD19, a response regulator of transmembrane signals during B cell activation[128]. CD35 binds surface-bound C1q, C3b, C4b, MBL and the inactivated form of C3b (iC3b). CD35 cannot directly mediate phagocytosis, but the uptake of complement-opsonised targets under inflammatory conditions is greatly enhanced after CD35 crosslinking. B cells and FDCs recognize surface-bound C3d through CD21, resulting in a sustained B cell activation[129, 130]. CR3 (CD11b) and CR4 (CD11c) are expressed predominantly on myeloid cells and mediate phagocytosis of C3b opsonised targets.

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Chapter 2

The role of antibodies and B cells in the pathogenesis of EAE

Index

1. B cell-deficient $J_H^{-/-}$ mice are fully susceptible to MOG_{35-55}–induced EAE

μMT mice, in which the gene for the membrane μ-heavy chain is disrupted, are frequently used as a model for B cell-deficiency[1] and are widely used in EAE-studies[2-10]. Nonetheless, μMT mice have been shown to produce IgA and IgA-producing B cells casting doubt upon the applicability of this B-cell deficiency model[11]. On the other hand, $J_H^{-/-}$ mice, which have a targeted deletion of all J_H gene segments, completely lack Ig expression and mature B cells[12]. To determine the involvement of B cells in the induction of EAE, we immunized $J_H^{-/-}$ mice with MOG_{35-55} peptide emulsified in complete Freund's adjuvant (CFA) and 7dpi lymphocytes were challenged with either cognate Ag (Figure 1) or with mitogen (data not shown). We found that B cell-deficiency does not affect cognate antigen-driven lymphocyte proliferation (Figure 1A) or effector cytokine production (e.g. IFNγ/IL-17) (Figure 1B). Furthermore these data demonstrate that T cells in the $J_H^{-/-}$ mice are functionally intact and can be activated through conventional activation pathways. Furthermore the $J_H^{-/-}$ mice developed a similar chronic and sustained disease (Figure 2A, Table 1). Disease onset was equivalent in wt control BL/6 and B cell deficient mice. Patterns of inflammation and demyelination, as well as the total size of structural damage in the spinal cords (Figure 2B), revealed only minor differences between $J_H^{-/-}$ and wt control mice. Both displayed an influx of $CD3^+$ T cells and Mac-3^+ monocytes/macrophages into the CNS, which relatively reflected the severity of clinical disease. Wt mice display a significant portion of invading B cells, while the also susceptible B cell-deficient mice expectedly did not, implying that B cell invasion seen in wt mice is either an epiphenomenon or that invading B cells serve a regulatory function as suggested previously[2, 3, 13, 14].

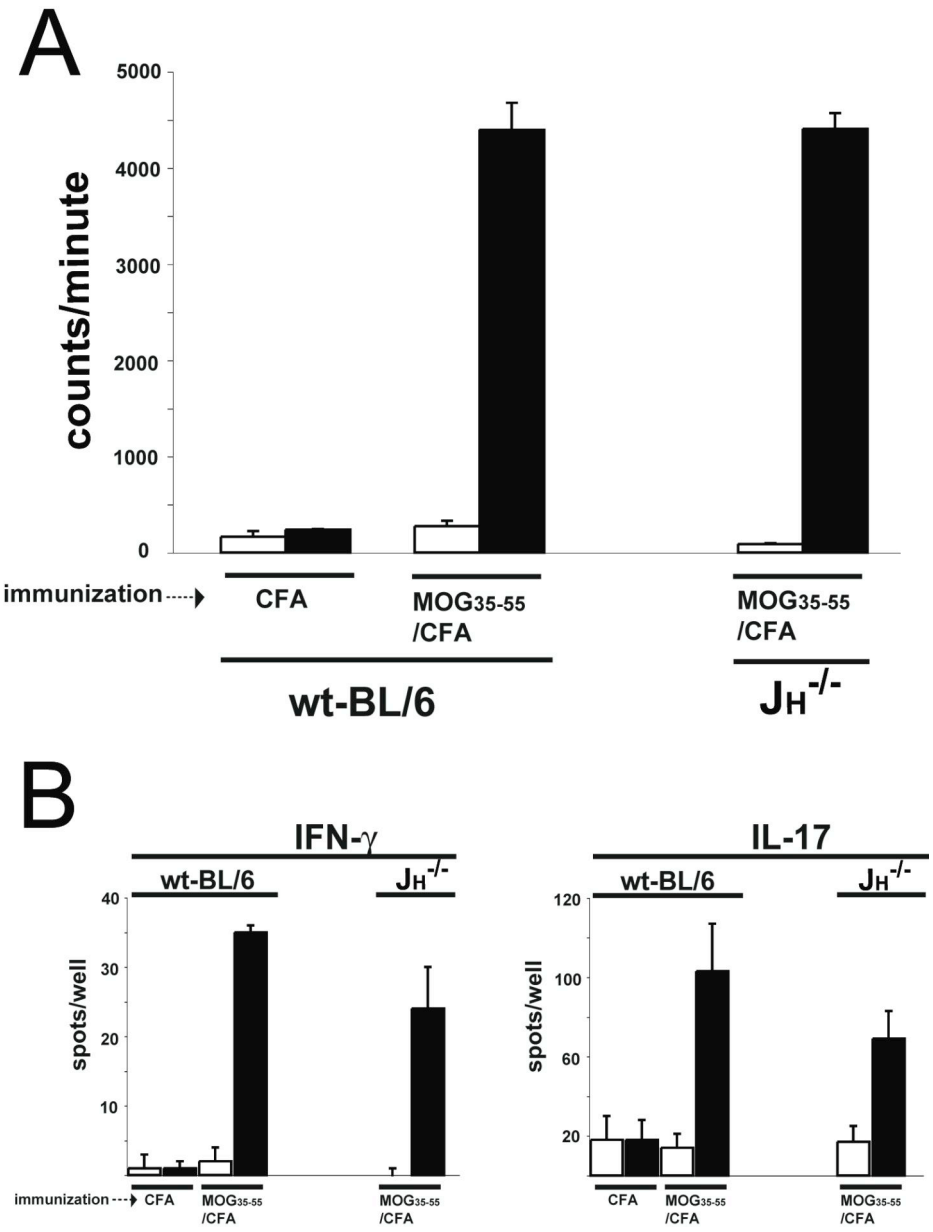


Figure 1 | B cells are not required for Ag-proliferation or effector cytokine production
Proliferation (A) and cytokine secretion (B) of lymphocytes from wt and $J_H^{-/-}$ mice immunized with MOG_{35-55}. (A) wt and $J_H^{-/-}$ mice were immunized with MOG_{35-55} emsulfied in CFA or CFA alone. Regional draining LNs were removed 7dpi and a single-cell suspension was restimulated with MOG_{35-55} (50µg/ml) in 96 well plates. T cell proliferation was determined by ^{3}[H]-thymidine incorporation in triplicate wells for 48 hours. Representative data from at least 2 experiments are presented as mean counts per minute (cpm) of triplicate cultures. Open bars represent control unstimulated cultures; filled bars are Ag stimulated cultures. (B) ELISPOT analyses for IFN-γ and IL-17 production from draining LN derived lymphocytes. Lymphocytes were prepared from wt and $J_H^{-/-}$ mice immunized with MOG_{35-55} 7dpi. IFN-γ and IL-17 secretion was elicited with restimulation with MOG_{35-55} (50µg/ml) (filled bars) or medium (open bars) for 24 hours. Data shown represent the mean of two individual experiments ± SEM.

mice	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt-BL/6	16/23	70%	12,2±2,4	2,2±0,9
$JH^{-/-}$	16/24	67%	12,3±2,8	2,6±1,0

[a)] diseased animals only

Table 1 | Clinical parameters of MOG_{35-55}-induced EAE in wt and B cell deficient $JH^{-/-}$ mice
Data are cumulated and averaged from four separate experiments.

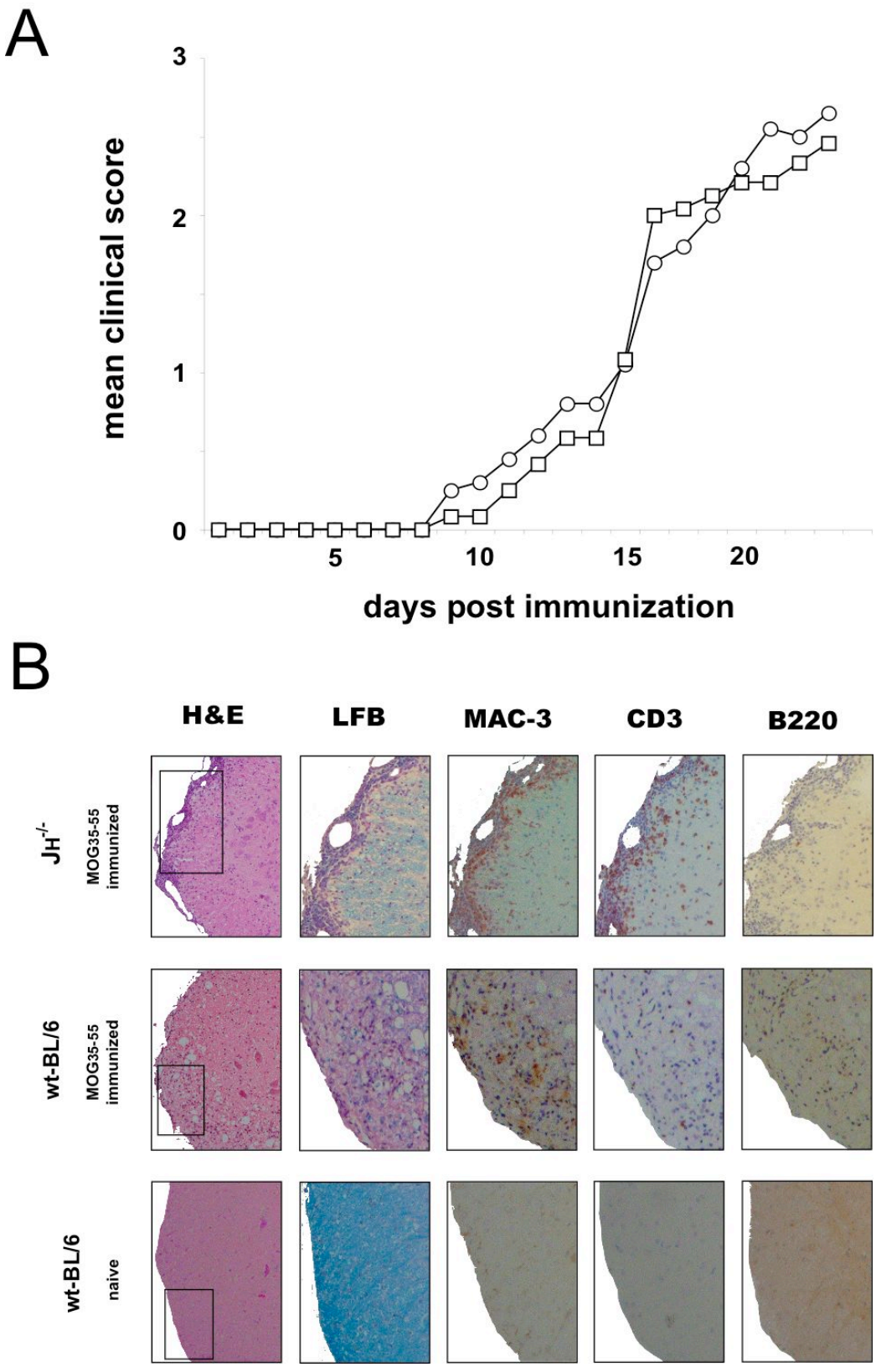


Figure 2 | B cell deficient JH$^{-/-}$ mice are susceptible to MOG$_{35-55}$ induced EAE
EAE was induced in (A) JH$^{-/-}$ (squares) and wt (circles) mice by immunization with MOG$_{35-55}$ in CFA and the mice were scored daily for clinical symptoms. The figure is a representative of at least four individual experiments (total n≥6/group). Statistical significance was determined using Student's t-test. B cell deficient JH$^{-/-}$ mice show CNS inflammation and demyelination after immunization with MOG peptide. (B) Paraffin-embedded spinal cord lateral column white matter of lumbar sections were prepared from perfused wt and J$_H^{-/-}$, animals at day 24 after induction of EAE with MOG$_{35-55}$. Hematoxylin and eosin (H&E) stains infiltrated cells, whereas luxol fast blue (LFB) shows myelin. Immunocytchemistry for CD3$^+$, MAC-3$^+$ and B220$^+$ subsets, shows infiltrating T cells, macrophages and B cells respectively.

2. B cells are critical for the induction of EAE by mouse and human MOG protein

Recent studies have indicated a role for different populations of APCs in the priming of naïve T cells in response to peptide vs. protein Ag and in this regard a crucial considerate to the origin of the protein Ag[15, 16]. While B cell-deficient mice developed EAE upon immunization with both human and rat MOG-peptide, recombinant full length human but not rat protein failed to induce EAE in µMT mice. This suggests that B cells are required to process and present Ag for proper T cell priming by different origin dependent mechanisms[6, 16, 17]. The recombinant MOG protein (rMOG) represents the mouse 121- respectively human 125-amino acids extracellular domain of MOG and contains the encephalitogenic MOG_{35-55} peptide (Figure 3)[18].

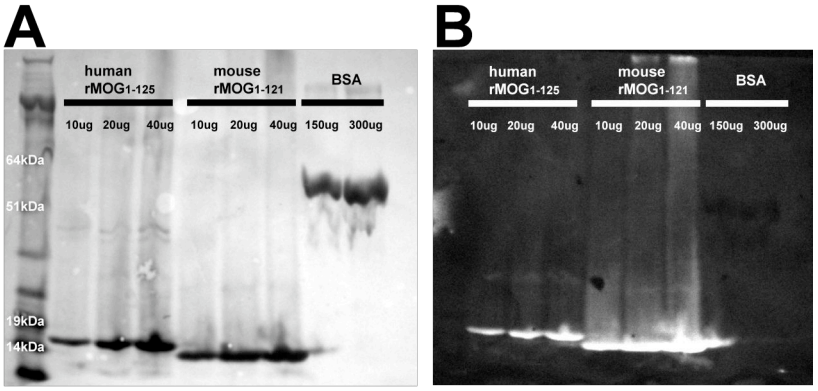


Figure 3 | Verification of the purified human and mouse rMOG by Western Blot analysis
The extracellular human $rMOG_{1-125}$ and mouse $rMOG_{1-121}$ protein were expressed in *Escherichia coli*, purified by Ni-chelate column and pooled protein containing fractions were dialyzed into acetate buffer and concentrated. Protein purity was assessed by (A) Ponceau-staining and (B) SDS-PAGE using an anti-MOG antibody (8-18C5) and a secondary goat-anti mouse-HRP antibody.

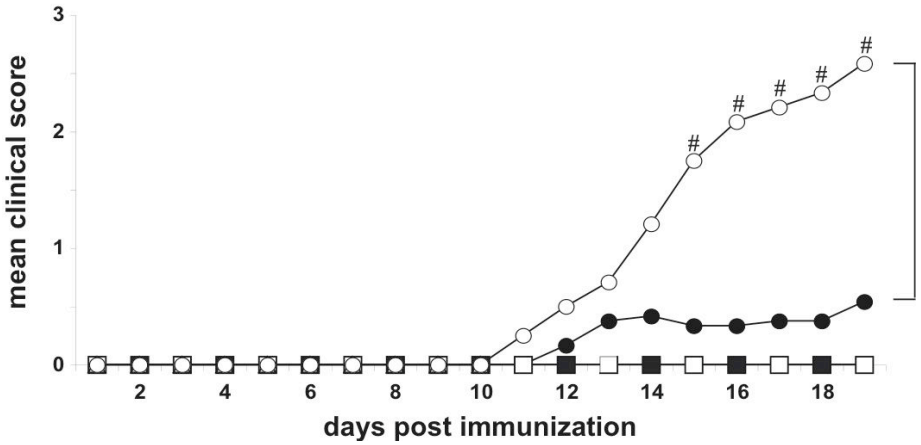
To assess whether B cells serve as APCs in the initiation of a T cell response to encephalitogenic protein antigens we immunized $J_H^{-/-}$ and wt mice with either a recombinant mouse MOG_{1-121} protein ($rMOG_{1-121}$) (Figure 4, Table 2) or recombinant human MOG_{1-125} ($rMOG_{1-125}$) (Figure 4) and followed the development of EAE.

Table 2 | Clinical parameters of mouse rMOG$_{1-121}$-induced EAE in wt and B cell deficient JH$^{-/-}$ mice
Data are cumulated and averaged from three separate experiments.

mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	mouse rMOG1-121	17/18	94%	12,4±2,2	2,42±0,6
$J_H^{-/-}$	mouse rMOG1-121	2/18	11%	10,5±0,7	2,25±0,4

[a)] diseased animals only

In contrast to the findings by Oliver et al.[16, 17], diseases induced with mouse MOG_{1-121} protein was significantly milder in $J_H^{-/-}$ compared to wt animals (Figure 4, Table 2) and we found $J_H^{-/-}$ mice to be fully resistance to the induction and progression of EAE after immunization with human MOG_{1-125} protein (Figure 4). These results indicate that B cells are of key importance for the induction of EAE by a large polypeptide.



mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	mouse rMOG1-121	12/12	100%	13,3±1,7	2,57±0,6
wt	human rMOG1-125	5/12	42%	13,0±0,7	2,10±0,5
$J_H^{-/-}$	mouse rMOG1-121	0/12	0%	n.d.	n.d.
$J_H^{-/-}$	human rMOG1-125	0/12	0%	n.d.	n.d.

[a)] diseased animals only

Figure 4 | B cell deficient mice are resistant or developed milder diseases to *protein-induced EAE*
wt (circles) and JH$^{-/-}$ (squares) mice were immunized with mouse rMOG$_{1-121}$ (open symbols) or human rMOG$_{1-125}$ (filled symbols). Mice were examined daily for clinical disease. The figures are representative of at least two separate experiments (total n≥6) and were analyzed for statistical significance using a Student's t-test (*<0,05, #<0,01). The table contains cumulated and averaged data from two separate experiments including the graph shown above.

3. MOG transgenic T cells proliferate in response to rMOG protein in $J_H^{-/-}$ mice

One explanation for the inability of MOG protein to induce EAE in B cell-deficient mice is that B cells play a role in the processing and presentation of the intact protein. It was previously demonstrated that mice lacking B cells (μMT) are impaired in their priming of T cells to protein but not peptide Ag[15]. Therefore we analyzed the capacity of $J_H^{-/-}$ mice to develop a protein or a peptide Ag-driven immune response. We adoptively transferred CFSE (carboxy fluorescein diacetate succinimide ester)-labeled TcR-transgenic (2D2) T cells, specific for MOG_{35-55} peptide[19] into either wt or $J_H^{-/-}$ mice. These cells had been labeled with CFSE, a fluorescent dye, which is passively incorporated into cells. Upon cell division the dye is divided equally between the daughter cells. Hence, every division reduces the dye intensity to the half. They were subsequently immunized with their cognate peptide (MOG_{35-55}), mouse $rMOG_{1-121}$ or human $rMOG_{1-125}$ protein emsulfied in CFA. After 4 days, proliferation of TcR Tg-T cells in lymph nodes (Figure 5) and spleen (data not shown) was determined by flow cytometry. As shown in Figure 5, $CD4^+$ TcR Tg-T cells proliferated to a similar extent in all $J_H^{-/-}$ groups in comparison to their wt control groups *in vivo* after immunization. No responses were observed in only CFA immunized mice. This data, therefore provide evidence in support of a preferential uptake and presentation of mouse in comparison to human rMOG *in vivo*, although all groups showed less proliferation by immunization with protein than with cognate peptide. These data indicate that B cell-deficient mice are capable of processing and presenting the rMOG protein.

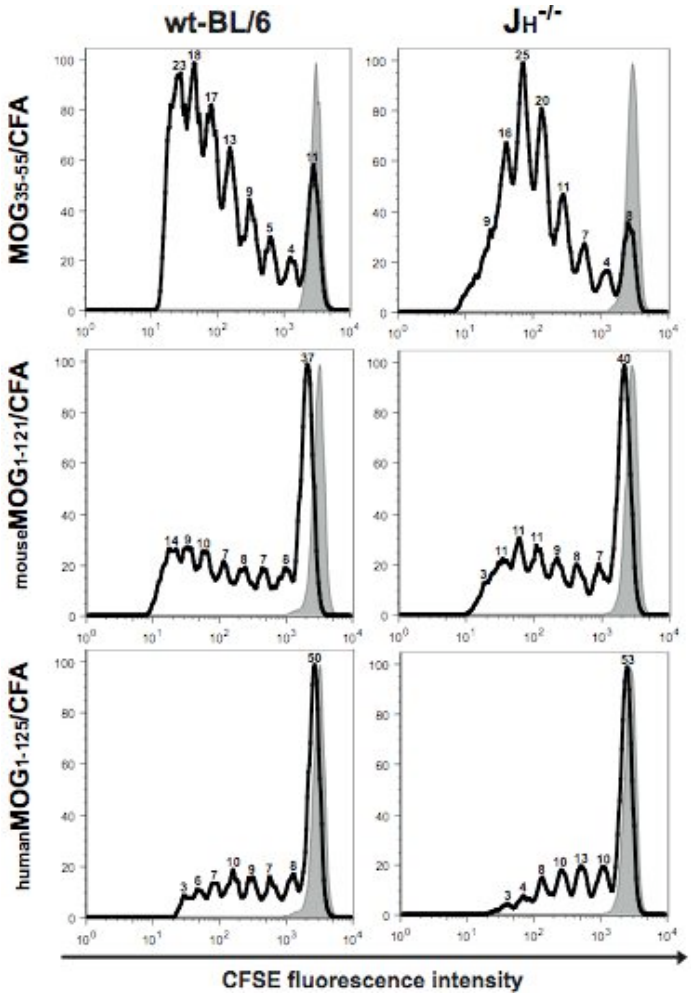


Figure 5 | *B cells are not required to process and present MOG proteins*
To assess the expansion of MOG-specific transgenic (2D2 Tg) T cells in the context of $J_H^{-/-}$ or wt host, $15x10^6$ lymphocytes from naïve 2D2 TCR transgenic mice were labeled with 10 µM CFSE and injected i.v. Recipients were subsequently immunized s.c. with $MOG_{35-55,}$ mouseMOG_{1-121} or humanMOG_{1-125} emulsified in CFA or CFA alone (grey). FACS analysis draining LNs was performed 4dpi. Gating on transgenic $V\alpha3.2^+$ cells permits to specifically follow the proliferation of the adoptively transferred T cells. Shown is a representative of two individual experiments (n ≥ 2).

4. Resistance of B cell$^{-/-}$ mice to MOG protein induced EAE does not depend on humoral anti-MOG response

Another possible explanation for the differential B cell dependence of MOG protein and MOG peptide is that immunization with encephalitogenic MOG_{35-55} peptide is a consequence of T cell epitope immunodominance[20, 21]. Furthermore the recognition of structural eptiopes of MOG could not only influence the binding of Abs *in vivo* but also result in different effector mechanisms for Abs pathogenicity[22]. To test if the

presence of conformational specific Abs contributes potentially to the development of EAE we first analyzed the levels of total Ig anti-MOG auto-Abs. Sera from MOG_{35-55}, MOG_{1-121} or Myelin immunized mice (Figure 6) were tested 12 dpi by using MOG_{35-55} coated (Figure 6A) or MOG_{1-121} coated (Figure 6B) ELISA plates. Surprisingly the polyclonal Abs present in serum of Myelin or MOG_{1-121} immunized wt mice do not recognize the linear Ag corresponding the short 35-55 peptide, which is also a part of MOG_{1-121} (Figure 6A). In contrast, in animals immunized with MOG_{35-55}, the repertoire of MOG reactive Abs was not restricted to linear epitopes (Figure 6B).

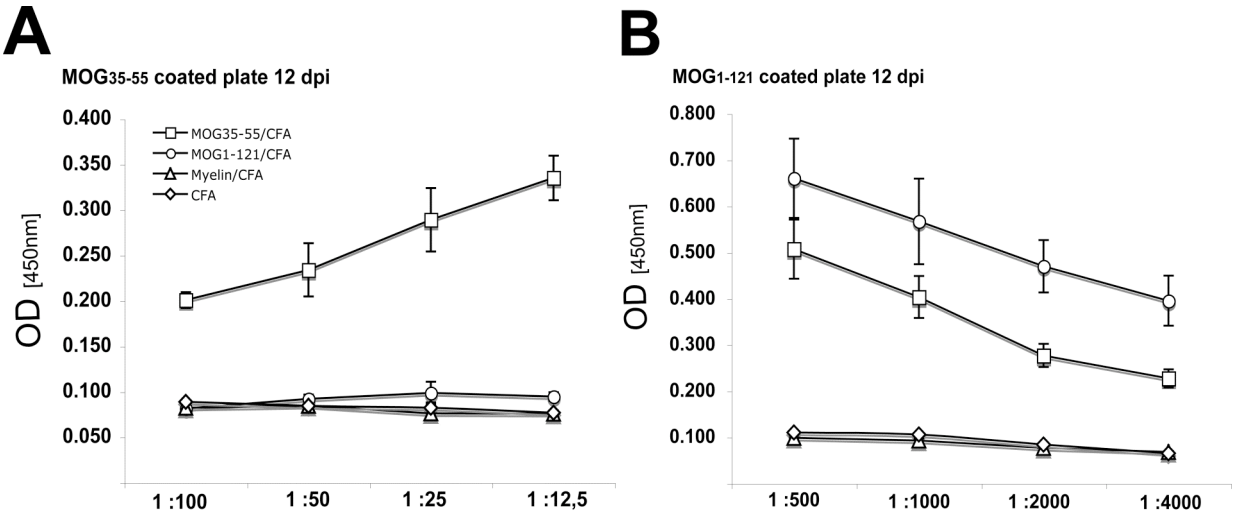


Figure 6 | *Igs from wt mice immunized with mouse MOG peptide, protein or myelin*
Serum antibody responses following MOG_{35-55} (200ug/mouse) (squares), MOG_{1-121} (200ug/mouse) (circles), Myelin (1:1 in CFA) (triangles) or CFA alone (diamonds). Anti-MOG_{35-55} (A) and anti-MOG_{1-121} specific (B) total Ig were assessed by ELISA using serum diluted 1:100, 1:50, 1:25 , 1:12,5 or 1:500, 1:1000, 1:2000, 1:4000 respectively. All sera were pooled from two mice 12 days post immunization. Values represent mean OD_{450nm} results (± SEM).

The MOG-specific mouse hybridoma 8-18C5 secretes a conformation-dependent anti-MOG mAb that can mediate demyelination *in vivo* in animals with EAE[20, 23, 24]. The MOG-specific Ab response is complex and recognizes both linear and conformation-dependent epitopes. The demyelinating component of this response is directed to conformation-dependent epitopes present on the extracellular Ig-like domain of the protein, whereas Abs recognizing linear MOG peptides are unable to

bind to the native protein and are unable to initiate demyelination *in vivo*[20, 23]. To investigate whether the role of anti-MOG protein Ab is in the priming of encephalitogenic T cells, or if these conformational anti-MOG Abs are able in trafficking of encephalitogenic T cells into the CNS, $J_H^{-/-}$ mice previously immunized with MOG_{35-55} peptide or MOG_{1-121} protein were treated with the monoclonal anti-MOG antibody (mAb) (8-18C5) (Figure 7). We observed that the systemic application of the anti-MOG antibody starting at the time of immunization increased clinical signs of EAE in both MOG_{35-55} peptide immunized wt and $J_H^{-/-}$ mice. We also noticed that the injection of the anti-MOG Abs did not lead to disease exacerbation in MOG_{1-121} protein immunized $J_H^{-/-}$ compared to wt mice (Figure 7).

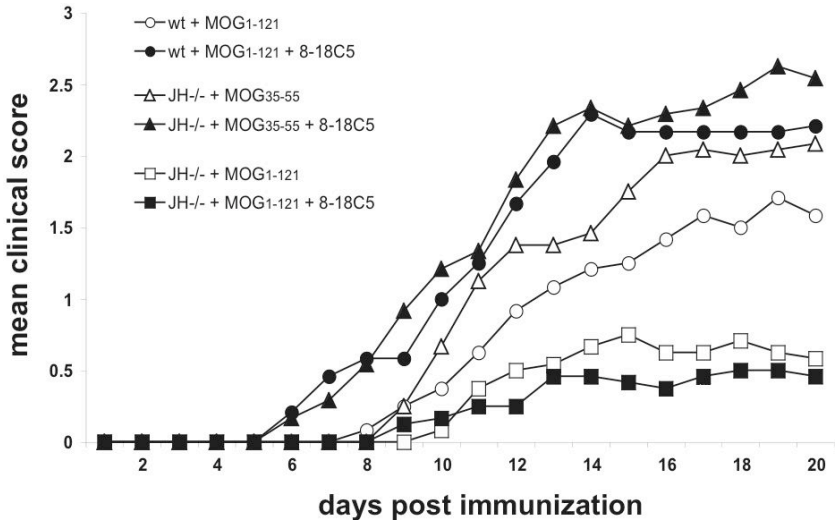
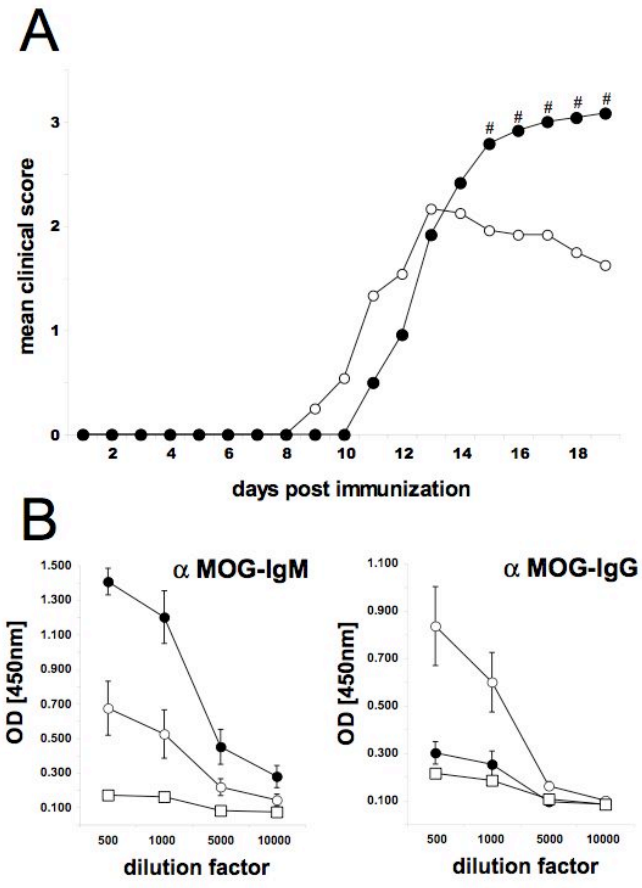


Figure 7 | *EAE resistance of $J_H^{-/-}$ mice is independent of a monoclonal demyelinating Abs*
wt and $J_H^{-/-}$ mice were immunized with MOG_{35-55} or MOG_{1-121} in CFA and at days -1, 3, 5 and 9 of immunization animals were given 150µg of either α-MOG mAb (filled symbols) or equal volume of PBS (open symbols) by i.v. injection into the tail vein. Mice were examined daily for clinical disease.

The germinal centres (GC) are the anatomical site where Ag activated B cells rapidly expand and differentiate into either plasma or memory B cells. GCs comprise of networks of follicular dendritic cells (FDCs), which play a vital role in the generation of a high affinity Ab-response through somatic hypermutation and isotype switching[25]. To dissect the relative contributions of high affinity autoreactive Abs in EAE, we

treated wt mice with Lymphotoxin-beta-receptor (LTβR)-Fc fusion protein, which has been shown to disrupt the formation of tertiary lymphoid tissues[26, 27] and assessed them for the development of EAE. The LTβR-Fc treated mice, although showed a similar disease onset, nevertheless they developed a more severe monophasic EAE compared to the human IgG treated control mice (Figure 8A Table 3). In addition, we analyzed the level of anti-MOG auto-Abs, in the sera from the LTβR-Fc treated and the control mice, by ELISA using MOG_{35-55} (Figure 8B). As expected, the LTβR-Fc treated mice weren`t able to generate high affinity IgGs against MOG but interstingly it seemed so that they try to compensate this inability by an increased secretion of anti MOG IgM. Taken together, our data clearly demonstrate that B cell activation and the generation of high-affinity Abs is not obligatory for EAE development and progression.



mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	PBS	10/11	91%	11,6±2,3	2,53±0,5
wt	LTβR-Fc	11/11	100%	11,4±1,0	3,15±0,1

[a)] diseased animals only

Figure 8 | The *lack of polyclonal anti-MOG IgGs has no effect on disease susceptibility*
(A) EAE was induced by s.c. immunization with MOG_{33-55} in LTβR-Fc treated (filled circles) and untreated (closed circles) wt mice. Results are representative of 2 independent experiments ($n \geq 5$ mice/group). (B) Anti-MOG IgM and IgG Ab ELISA from sera of diseased LTβR-Fc treated (filled circles), untreated wt (open circles) and unimmunized mice (square) (19dpi). Shown is the average of 4 mice per group ± SEM. The table contains cumulated and averaged data from two separate experiments including the graph shown aside.

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Chapter 3

The contribution of Fc receptors and complement system to EAE

Index

1. Deficiency in FcRγ delays onset and decreases severity of EAE, independent of Ab binding

It is well documented that the FcR-associated signaling subunit, termed the common γ-chain (FcRγ) is important in the development of EAE as FcRγ$^{-/-}$ mice are EAE resistant[1-3], even though they developed comparable levels of MOG-specific Abs[1, 2]. To assess whether this EAE-resistance is due to the absence of FcR bearing cells to cause demyelination of Ab-coated myelin structures, we initially immunized FcRγ$^{-/-}$ and wt mice (both on the C57BL/6 background) with MOG_{35-55} in CFA and evaluated clinical disease development. Consistent with the findings by Lock et al. in BL/6-129PF2 strain[3], we found that while wt mice developed progressive paralysis, FcRγ$^{-/-}$ mice were relatively resistant to EAE and developed a significantly ($p<0.01$) milder disease with delayed onset (Figure 1A, Table 1).

The relative EAE resistance of FcRγ$^{-/-}$ mice is in clear conflict with the finding that mice lacking all Abs develop severe EAE[4-9], raising the question as to whether the FcRγ-deficiency functions independent of Abs. To this end, we crossed B cell deficient $J_H^{-/-}$ mice with FcRγ$^{-/-}$ mice. $J_H^{-/-}$ mice, which in contrast to the widely used μMt mice (targeted deletion of μ heavy chain), are completely deficient in mature B cells and Igs[10, 11]. After induction of EAE, we found that similar to FcRγ$^{-/-}$ mice, FcRγ$^{-/-}$/$J_H^{-/-}$ mice displayed a delayed disease onset and decreased severity (Figure 1B, Table 2), demonstrating that the EAE-resistance of FcRγ$^{-/-}$ mice functions independently of B cells and their products. Immunohistochemical analysis revealed significantly reduced pathological changes in the FcRγ$^{-/-}$ and FcRγ$^{-/-}$/$J_H^{-/-}$ when compared to wt mice. The FcRγ$^{-/-}$/$J_H^{-/-}$ mice developed only mild demyelination as

assessed by Luxol-fast blue staining (LFB) and a substantial decrease in cellular infiltration (H&E) when compared to wt mice (Figure 11).

In addition, we analyzed the levels of MOG auto-antibodies and antibody responses to keyhole limpet hemocyanin immunization (KLH) by ELISA. Sera from both the FcRγ$^{-/-}$ and the wt mice immunized with either MOG_{35-55} (Figure 1C) or $rMOG_{1-121}$ (Figure 1D) had comparable levels of total Igs, IgG and IgM antibodies. This was also the case for KLH as a representative protein antigen (Figure 1E), demonstrating that Abs against myelin raised after immunization do not contribute significantly to the development of EAE.

Table 1. Corresponding to Fig. 1*A*

Mice	Disease incidence, n (%)	Avg day of onset ± SEM*	Mean max clinical severity± SEM*
WT BL/6	6 of 6 (100)	12.5 ± 3.2	2.6 ± 0.7
FcRγ$^{-/-}$	1 of 6 (17)	17 ± 0	3 ± 0

* diseased animals only

Table 2. Corresponding to Fig. 1*B*

Mice	Disease incidence, n (%)	Avg day of onset ± SEM*	Mean max clinical severity± SEM*
WT BL/6	6 of 6 (100)	14.8 ± 5.1	2.21 ± 0.9
$J_H^{-/-}$/FcRγ$^{-/-}$	1 of 6 (17)	16 ± 0	2.75 ± 0

* diseased animals only

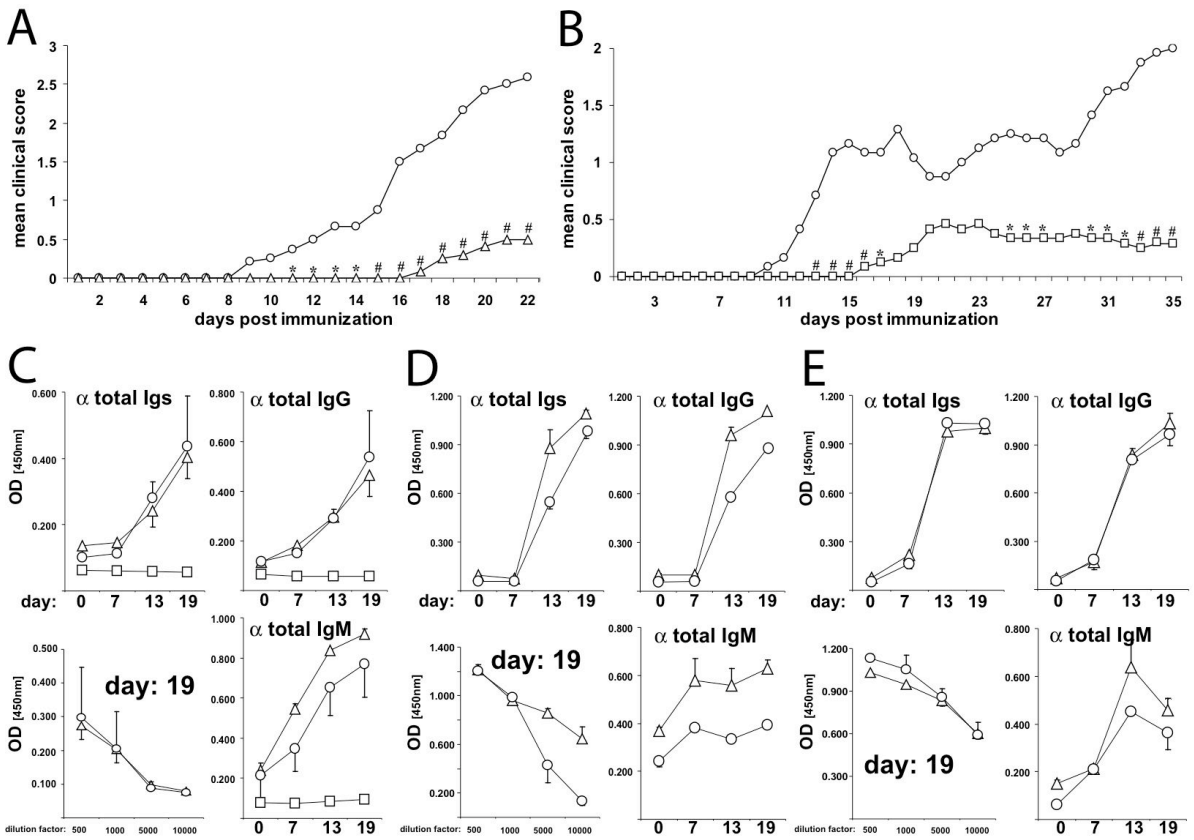


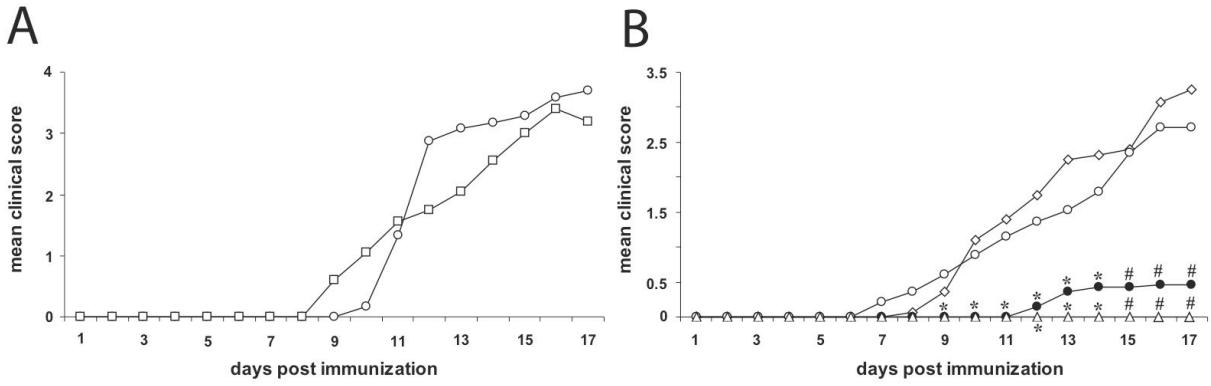
Figure 1 | *The loss of FcRγ attenuates EAE development independent of B cell function.*
EAE was induced in (A) FcRγ$^{-/-}$ (triangles) and wt (circles) or (B) $J_H^{-/-}$/FcRγ$^{-/-}$ (squares) and wt (circles) mice by immunization with MOG_{35-55} in CFA and the mice were scored daily for clinical symptoms. The figure is a representative of at least three individual experiments (total n≥6/group). Statistical significance was determined using Student's t-test (*<0,05, #<0,01). Panels (C-E) display the relative analysis of antibody responses from immunized FcRγ$^{-/-}$ (triangles), $J_H^{-/-}$/FcRγ$^{-/-}$ (squares) and wt controls (circles). Serum antibody responses following (C) MOG_{35-55} (200ug/mouse), (D) $rMOG_{1-121}$ (200ug/mouse) and (E) KLH (200ug/mouse) (emsulfied 1:1 in CFA) on day 0, 7, 13 and 19 post immunization (dpi). MOG- and KLH-specific total Ig, IgG and IgM levels were assessed by ELISA using serum diluted 1:1000. For 19 dpi sera were titrated 1:500, 1:1000, 1:5000 and 1:10000. Values represent mean OD_{450nm} results (± SEM) from 4 mice of two different experiments.

2. Peripheral leukocytes but not CNS resident cells are required for FcRγ-mediated disease development

CNS-resident microglia are widely held to be central to the development of CNS lesions[12-16] and their expression of FcγRs (FcγRI, FcγRII and FcγRIII) is thought to mediate Ab-induced tissue destruction[15, 17]. The use of BM chimeric mice allows to selectively manipulate the genotype of the peripheral haematopoietic immune system while sparing host-derived CNS resident cells such as CNS parenchymal microglia, astroglia and macrophages, which are not significantly repopulated after BM reconstitution[13, 18]. In contrast to the CNS parenchyma, the genotype of the systemic immune compartment and that of perivascular cells can be exchanged with that of donor-derived haematopoietic cells[13, 14, 19].

To determine the role and function of FcRs expressed by CNS-resident cells, we generated a series of BM chimeric mice and induced EAE via active immunization with MOG_{35-55}. The absence of FcRγ from the radio-resistant (microglia) compartment (wt → $FcR\gamma^{-/-}$) did not alter the disease course when compared to control mice (wt → wt) (Figure 2A), whereas the deletion of FcRγ from the systemic immune compartment ($FcR\gamma^{-/-}$ → wt) resulted in reduced clinical disease (Figure 2B). We conclude that FcRγ expression by CNS resident cells does not influence the course of clinical disease or histopathological changes (data not shown) associated with CNS inflammation. Considering the potential direct involvement of FcRγ signaling in lymphocyte activation[20-23], we wanted to assess whether lymphocyte function *in vivo* is directly affected by the loss of FcRγ or whether accessory cells require FcRγ for the development of autoimmunity. In order to narrow down the

FcRγ-bearing cell influencing EAE, we constructed mixed BM-chimeras in which the FcRγ-lesion is restricted to either lymphocytes or to all other leukocytes. To restrict genetic deficiency to lymphocytes only, the BM inoculum consisted of 25% RAG1$^{-/-}$ BM cells supplemented with 75% BM cells from FcRγ$^{-/-}$ mice. Control groups received either 100% wt, 100% RAG1$^{-/-}$ or 100% FcRγ$^{-/-}$ BM cells. As expected the groups reconstituted with 100% RAG1$^{-/-}$ or 100% FcRγ$^{-/-}$ were resistant or developed mild EAE, respectively (Figure 2B). Mice bearing FcRγ$^{-/-}$ lymphocytes but carrying normal accessory leukocytes (FcRγ$^{-/-}$ + RAG$^{-/-}$ → wt) were fully susceptible to EAE and indistinguishable from the wt → wt control group (Figure 2B). Taken together, our data demonstrate that FcRγ is vital for the development of EAE and it must exert its primary effect through accessory cells such as mono- and polymorphonucleated phagocytes, dendritic cells (DCs) or natural killer cells (NK cells) but not through lymphocytes directly.



group	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt → wt	5/5	100%	10,6±1,9	3,45±0,1
wt → FcRγ$^{-/-}$	6/6	100%	11,3±0,8	3,71±0,6

[a)] diseased animals only

group	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt → wt	6/7	86%	10,8±3,6	3,21±0,5
FcRγ$^{-/-}$ → wt	1/6	17%	12±0	3,25±0
Rag1$^{-/-}$ → wt	0/4	0%	n.d.	n.d.
FcRγ$^{-/-}$ + Rag1$^{-/-}$ → wt	6/7	86%	9,8±1,7	3,79±1,1

[a)] diseased animals only

Figure 2 | *FcRγ-bearing accessory cells restore EAE susceptibility in BM chimeras.*
(A) BM chimeric mice in which the FcRγ deficiency was restricted to CNS-resident cells were generated by transferring 2x10^7 wt-BM cells into lethally irradiated FcRγ$^{-/-}$ (wt → FcRγ$^{-/-}$, open circles) and wt mice (wt → wt, open squares) as a control group. (B) Irradiated wt mice were reconstituted with BM cells from RAG1$^{-/-}$ mice (RAG$^{-/-}$ → wt, open triangles), FcRγ$^{-/-}$ deficient mice (FcRγ$^{-/-}$ → wt, filled circles), wt mice (wt → wt, open circles) and a 1:4 mixture of FcRγ$^{-/-}$ and RAG1$^{-/-}$ BM cells (FcRγ$^{-/-}$ + RAG$^{-/-}$ → wt, open diamonds). 8 weeks later, reconstituted BM chimeras (≥ 6/group) were actively immunized with MOG$_{35-55}$ in CFA and clinical disease development was assessed daily. The table corresponds to the graph shown above. The figure is representative of at least two separate experiments (total n≥ 10). Statistical significance was determined using Student's t-test (*<0,05, #<0,01).

To determine whether loss of FcRγ lesions the capacity of APCs to drive the expansion of encephalitogenic T cells, FcRγ$^{-/-}$ and wt mice were immunized with MOG and 7dpi lymphocytes were challenged with either cognate Ag or with mitogen. Interestingly, we found that FcRγ-deficiency does not affect cognate antigen-driven lymphocyte proliferation (Figure 3A) or cytokine production (e.g. IFNγ/IL-2) (Figure 3B). To corroborate the capacity of FcRγ$^{-/-}$ mice to develop a protein/peptide Ag-driven immune response we adoptively transferred CFSE-labeled TcR-transgenic (2D2) T cells, specific for MOG_{35-55}-peptide[24] into either wt or FcRγ$^{-/-}$ mice. They were subsequently immunized with their cognate peptide (MOG_{35-55}) or MOG_{1-121} protein emulsified in CFA. After 4 days, proliferation of TcR Tg-T cells in spleen and LNs was determined by flow cytometry. As shown in Figure 3C, all groups displayed similar proliferation *in vivo* after antigen challenge.

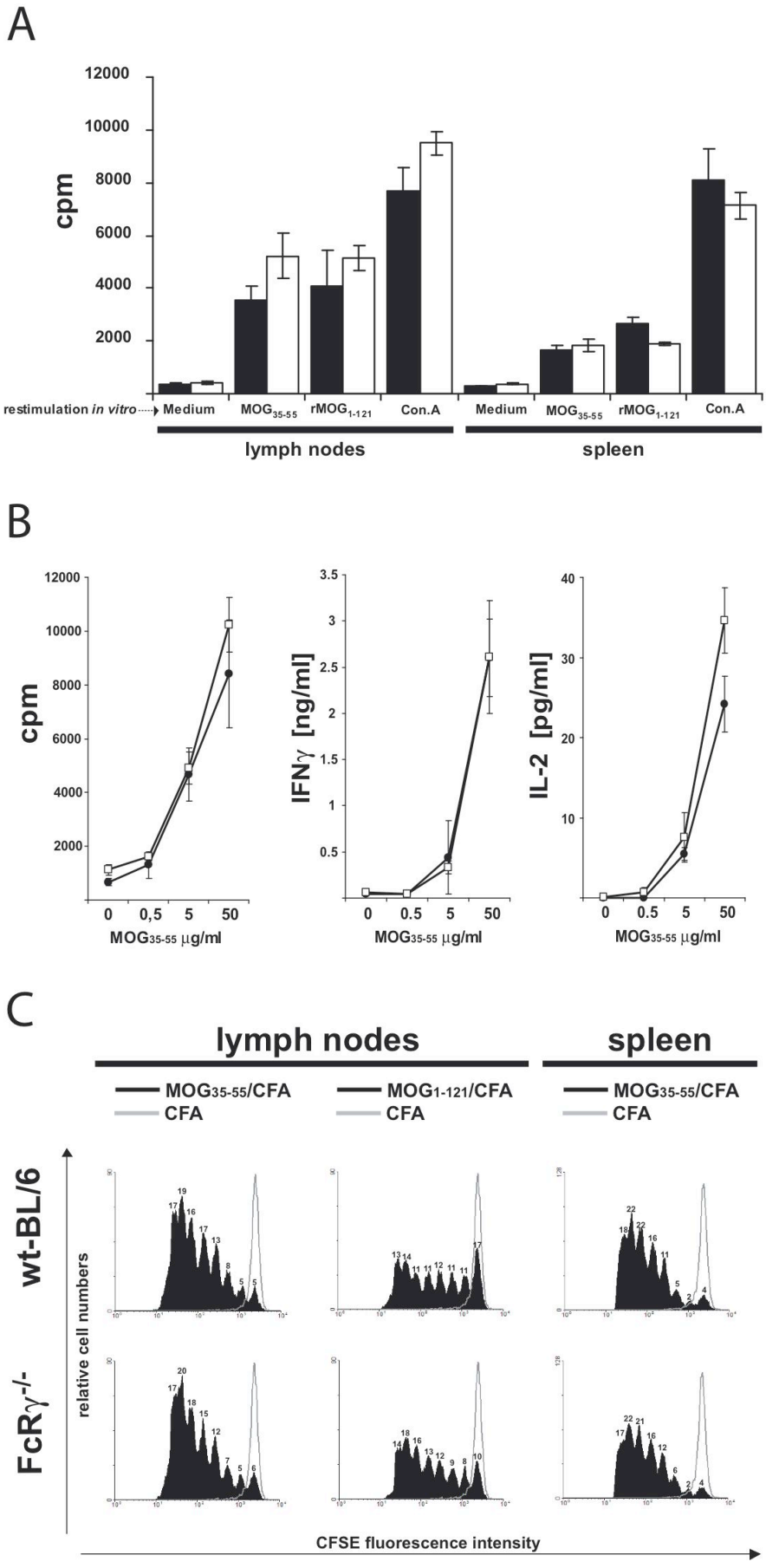


Figure 3 | ***Encephalitogenicity but not Ag-proliferation and cytokine production are dependent on FcRγ.***
(A) wt (black) and FcRγ$^{-/-}$ (white) mice were immunized with MOG_{35-55} emsulfied in CFA. Regional draining LNs and spleens were removed 7dpi and a single-cell suspension was restimulated with MOG_{35-55} (50µg/ml), $rMOG_{1-121}$ (50µg/ml) or ConA (5µg/ml) in 96 well plates. T cell proliferation was determined by ^{3}[H]-thymidine incorporation in triplicate wells for 48 hours. Representative data from at least 3 experiments are presented as mean counts per minute (cpm) of triplicate cultures. (B) Lymphocytes were prepared from wt (filled circles) and FcRγ$^{-/-}$ (open squares) mice immunized with MOG_{35-55} 7dpi. IFN-γ and IL-2 secretion was elicited with different doses of MOG_{35-55} for 24 hours and measured by ^{3}[H]-thymidine incorporation and ELISA. Data shown represent the mean of two individual experiments ± SEM. (C) To assess the expansion of MOG-specific transgenic (2D2 Tg) T cells in the context of FcRγ$^{-/-}$ or wt host, 25x10^{6} lymphocytes from naïve 2D2 TCR transgenic mice were labeled with 10 µM CFSE and injected i.v. Recipients were subsequently immunized s.c. with MOG_{35-55}, MOG_{1-121} emulsified in CFA or CFA alone. FACS analysis of spleen and draining LNs was performed 4dpi. Gating on transgenic Vα3.2^{+} cells permits to specifically follow the proliferation of the adoptively transferred T cells. Shown is a representative of two individual experiments (n ≥ 2).

3. Loss of FcRγ diminishes inflammation during the effector phase of EAE

A possible explanation for the observed function of FcRγ on accessory cells is that FcRγ signaling is important for the capacity of APCs to properly prime and polarize naïve T cells towards an effector phenotype. To asses the response of wt and FcRγ$^{-/-}$ mice to mitogen stimulation and to investigate functionality of the innate immune response in FcRγ$^{-/-}$ mice, we stimulated lymphocytes obtained from naïve wt or FcRγ$^{-/-}$ mice *in vitro* with either concavilin A (ConA) or lipopolysaccharide (LPS) and measured proliferation (Figure 4A) as well as the secretion of IFN-γ and IL-2 (Figure 4B). We did not observe any significant differences between the responses of wt or FcRγ$^{-/-}$ mice (Figure 4).

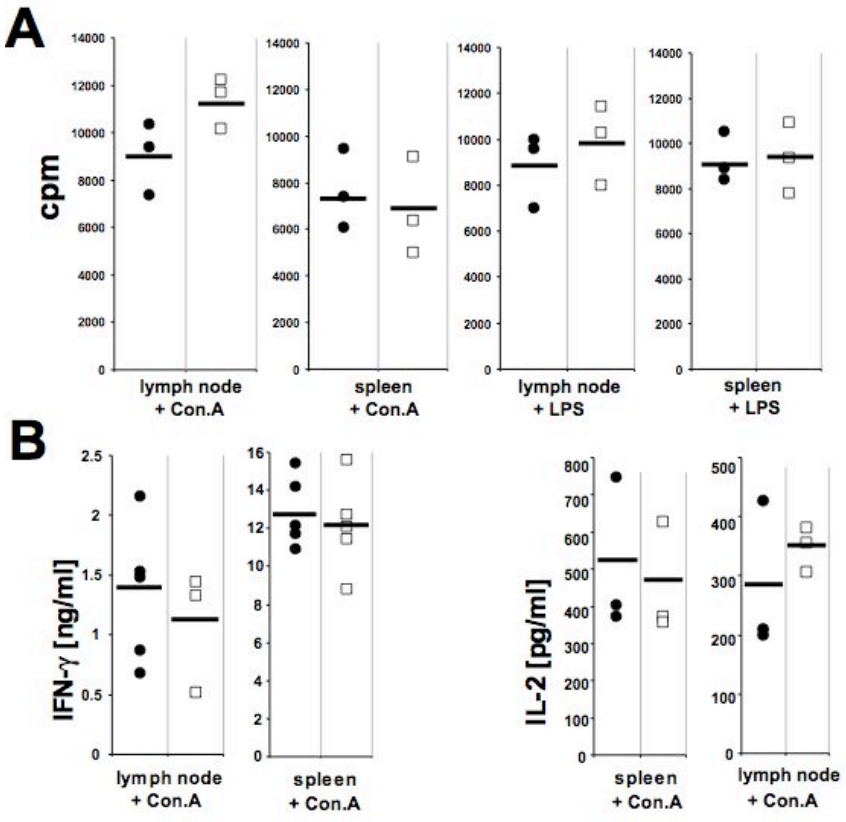


Figure 4 | ***Ex vivo proliferative capacity and secretion ability of naive FcRγ$^{-/-}$ lymphocytes.***
(A) Measuring of proliferation and (B) ELISA assessing IFN-γ and IL-2 secretion by naïve wt and naïve FcRγ$^{-/-}$ LN and spleen derived cells. They were stimulated for 20 hours with 5µg/ml ConA or 5µg/ml LPS. ^{3}[H]-thymidine was added to the culture 18 hours prior to measuring proliferation in counts per minute (CPM). Data shown represent the mean of two individual experiments.

We next generated BM-chimeras by transferring 1:1 ratio of FcRγ chain deficient BM cells (CD45.2) and CD45.1-congenic wt BM cells into lethally irradiated wt recipients. Wt-CD45.2 and wt-CD45.1 BM-chimeras were generated as a control. The mice were immunized with MOG_{35-55} and after 7 days splenocytes were removed and challenged *in vitro* with MOG. Neither MOG_{35-55}-peptide nor $rMOG_{1-121}$-protein restimulated FcRγ deficient $CD4^+$ cells differ in proliferation from $CD4^+$ cells with intact FcRγ (Figure 5).

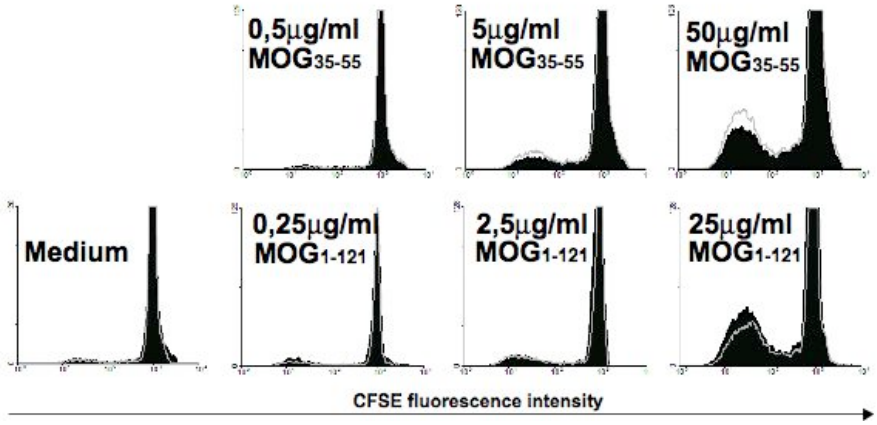


Figure 5 | ***Antigen specific expansion despite FcRγ-chain deficiency.***
The generated wt-Ly5.1 : FcRγ-Ly5.2 BM-chimeric mice, whose immune system contain a 1:1 mix of FcRγ deficient-Ly5.2 (grey line) and wt-Ly5.1 cells (black filled) were immunized with MOG_{35-55} and after 7 days splenocytes were challenged *in vitro* with different concentrations of either MOG_{35-55} or MOG_{1-121} (0.5, 5, 50 µg/m).

In a complementary approach to determine the APC capacity of $FcR\gamma^{-/-}$ DCs, we exposed immature (Figure 6A) or LPS matured (Figure 6B) bone marrow (BM)-DCs, derived from wt or $FcR\gamma^{-/-}$ mice, to p11-peptide and co-cultured them with TcR transgenic T cells (SMARTA). After 24 hours, proliferation was measured by using ^{3}[H] thymidine incorporation. No significant difference in T cell priming between the groups was observed regardless of the degree of DC maturation (Figure 6). The results suggested that FcRγ is not involved in APC maturation or Ag-presentation and that priming and expansion of autoreactive T cells is undisturbed.

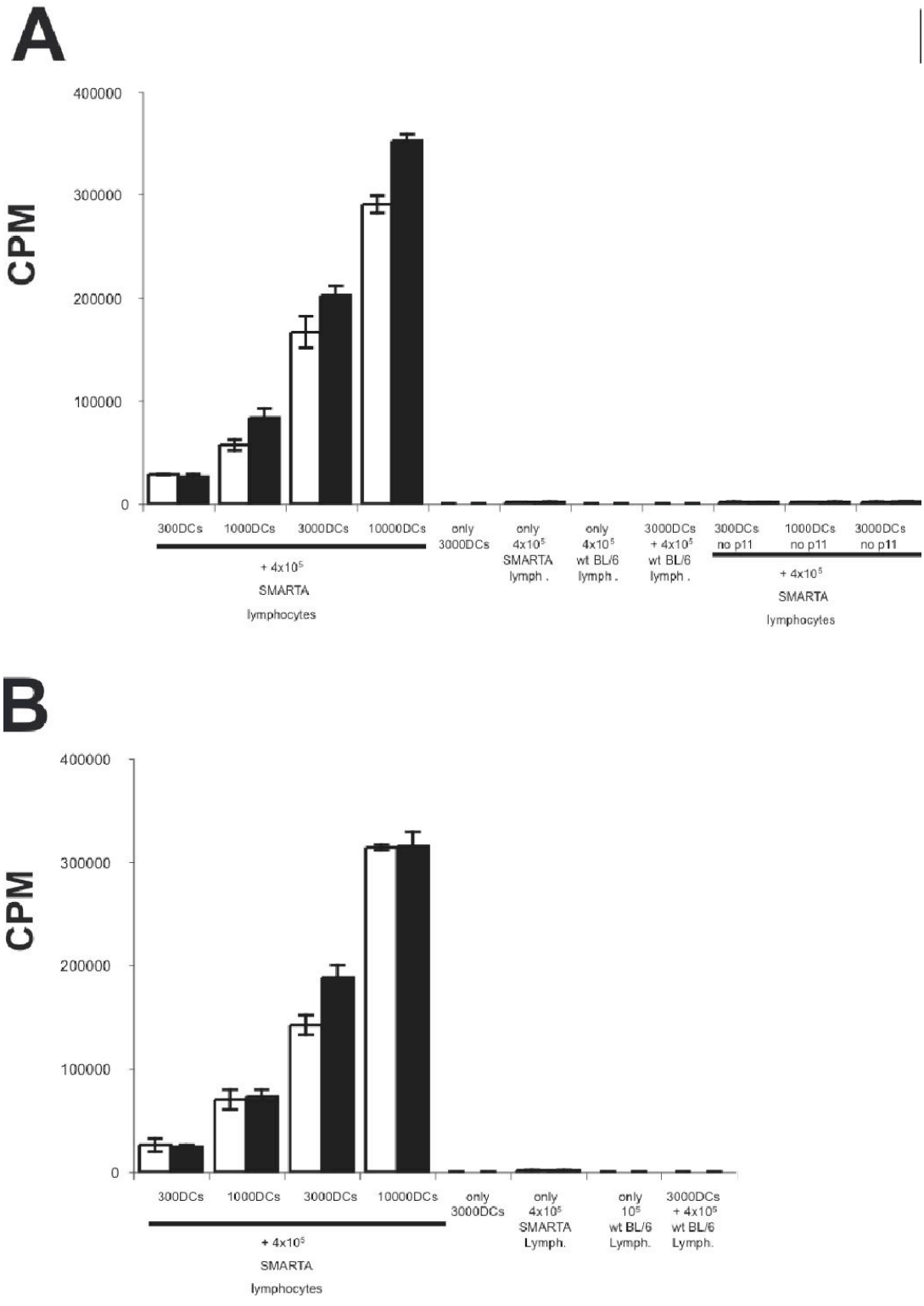


Figure 6 | *Antigen specific expansion despite FcRγ-chain deficient BM-DCs.*
(A) Immature bone marrow (BM)-derived DC's were generated, by addition of GM-CSF, from wt (white) and FcRγ$^{-/-}$ (black) mice, (B) matured with 10μg/ml LPS o.N. and subsequently pulsed with 1 μg/ml SMARTA peptide (p11). 4x10^5 p11-specific splenic-derived CD4$^+$ T cells were obtained from naïve SMARTA-Tg mice and co-cultured with the peptide-pulsed (1μg/ml), irradiated (2000 rads) DC's for 24h when proliferation was assessed by ^{3}[H]-thymidine incorporation in counts per minute (CPM). Data shown represent the mean of two individual experiments ± SEM.

In order to check the ability of myeloid cells to reach the CNS tissue, we generated mixed bone marrow chimeras by transferring CD45 congenic wt and FcRγ$^{-/-}$ bone marrow into wild-type recipients and evaluated the capacity of myeloid population to invade the CNS during disease. We first confirmed that the mice had a 1:1 ratio of wt and FcRγ$^{-/-}$ hematopoetic cells in peripheral blood and spleens. After EAE induction, we found both myeloid populations in the CNS at a ratio of 1:1, indicating that there are no difference between the two genotypes of myeloid cells (data not shown).

To assess the role and function of FcRγ during the effector phase of EAE we determined the susceptibility of FcRγ$^{-/-}$ mice to EAE induced by adoptive transfer of a population of encephalitogenic lymphocytes. Fully primed and activated encephalitogenic wt T cells induced EAE in wt recipients, yet surprisingly they were incapable of inducing clinical EAE in FcRγ$^{-/-}$ hosts (Figure 7). This finding again shows that FcRγ signaling is crucial for the actions of the accessory cell compartment during the effector phase of the disease.

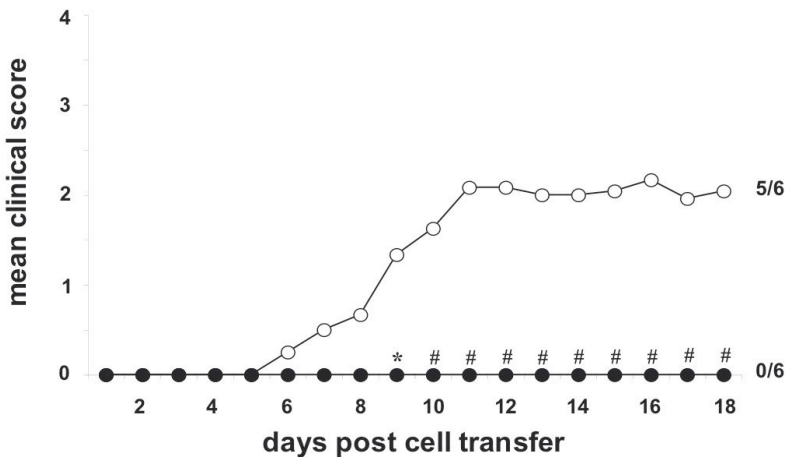
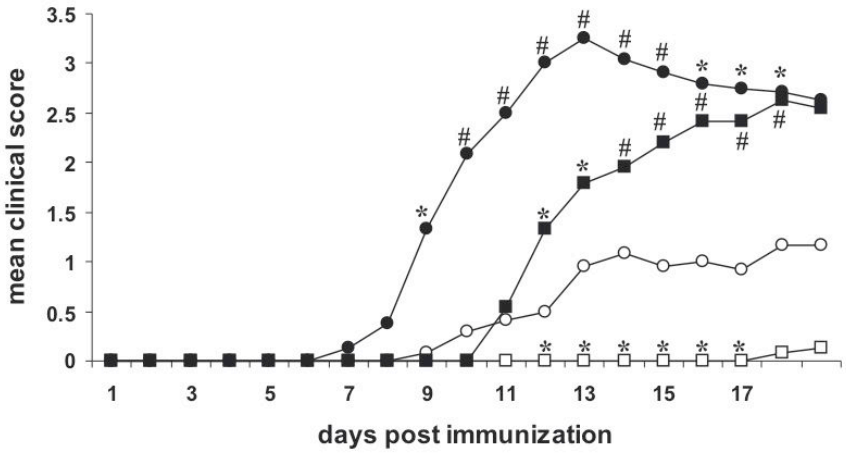


Figure 7 | *Deletion of FcRγ abrogates encephalitogenicity of effector T cells in EAE.* FcRγ$^{-/-}$ (filled circles) and wt (open circles) mice received a single injection of 30x10^6 encephalitogenic MOG-reactive wt-derived lymphocytes (prepared as described above). 3 hours later the mice received 200ng PT i.p.. Subsequently, the mice were examined daily for clinical disease. Data show the mean EAE score and the figure is a representative of three separate experiments (total n=18). Statistical significance was determined using Student's t-test (*<0,05, #<0,01).

4. Exacerbation of EAE after injection of demyelinating anti-MOG mAbs is complement-dependent but not driven by ADCC

After disconnecting the actions of FcRγ from the function of anti-MOG Abs generated through immunization with MOG, we were pressed to consolidate our findings with the fact that α-MOG Abs have been shown to exacerbate EAE[25-27]. We observed that the systemic application of monoclonal anti-MOG antibody (mAb) (8-18C5) starting at the time of immunization rapidly increased clinical signs of EAE in both wt and FcRγ$^{-/-}$ mice, clearly demonstrating that Ab-mediated exacerbation of EAE is independent of FcRγ (Figure 8). We noticed that this exacerbation of clinical EAE did not affect the delayed onset of clinical symptoms characteristic for FcRγ$^{-/-}$ mice, again supporting the notion that the function of FcRγ in EAE is unrelated to Ab binding and FcRγ signaling may contribute to the preclinical phase of the disease. The anti-MOG-mAb caused strong demyelination and increased cellular infiltration of T cells, macrophages and B cells, when compared to mice treated with either isotype control Ab or PBS alone (Figure 12).

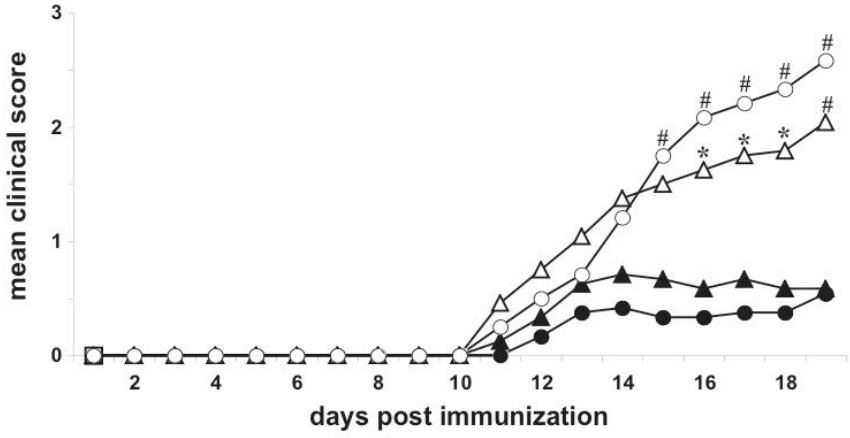
Numerous studies have documented deposition of complement components in MS lesions[28-30]. Anti-myelin Abs, which are potent complement activators, were also recently demonstrated in situ in MS[31, 32] and it was shown that injection of anti-MOG Abs into animals with acute EAE results in massive activation of complement in areas of demyelination[27]. We therefore investigated the relevance of the immune complex-dependent portion of the complement system in the Ab-mediated worsening of EAE by injection of αMOG mAbs into MOG immunized C1q$^{-/-}$ mice. C1q$^{-/-}$ mice developed EAE with the same onset and severity as the wt control group after immunization with either encephalitogenic MOG_{35-55} or recombinant mouse and human protein (Figure 9) and importantly the injection of the anit-MOG-mAb did not lead to disease exacerbation in C1q$^{-/-}$ compared to wt or FcRγ$^{-/-}$ mice (Figure 10).



mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	control	4/6	67%	11±1,8	2,19±1,9
wt	8-18C5	6/6	100%	8,3±1,2	3,29±0,8
FcRγ$^{-/-}$	control	1/6	17%	18±0	1±0
FcRγ$^{-/-}$	8-18C5	6/6	100%	12,5±1,6	2,83±0,4

[a)] diseased animals only

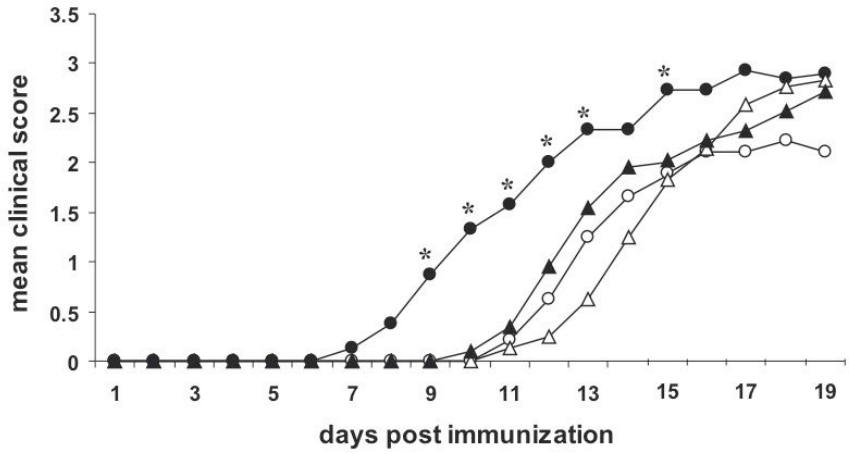
Figure 8 | *Demyelinating Abs drive the disease process in a FcR independent fashion.*
wt (circles) and FcRγ$^{-/-}$ (squares) mice were immunized with MOG_{35-55} in CFA and at days -1, 3, 5 and 9 of immunization animals were given 150µg of either α-MOG mAb (filled symbols) or equal concentration/volume of isotype control Ab or PBS (open symbols) by i.v. injection into the tail vein. Mice were examined daily for clinical disease and the table corresponds to the graph shown above. The figures are representative of at least two separate experiments (total n≥10) and were analyzed for statistical significance using a Student's t-test (*<0,05, #<0,01).



mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	mouse rMOG$_{1-121}$	12/12	100%	13,3±1,7	2,57±0,6
wt	human rMOG$_{1-125}$	5/12	42%	13,0±0,7	2,10±0,5
C1q$^{-/-}$	mouse rMOG$_{1-121}$	9/10	90%	12,0±2,0	2,61±0,5
C1q$^{-/-}$	human rMOG$_{1-125}$	5/9	55%	14,2±3,6	1,65±1,1

[a)] diseased animals only

Figure 9 | *Mouse and human rMOG induce EAE independent of classical complement mediated Abs response.*
wt (circles) and C1q$^{-/-}$ (triangles) mice were immunized with mouse $rMOG_{1-121}$ (open symbols) or human $rMOG_{1-125}$ (filled symbols). Mice were examined daily for clinical disease and the table corresponds to the graph shown above. The figures are representative of at least two separate experiments (total n≥10) and were analyzed for statistical significance using a Student's t-test (*<0,05, #<0,01).



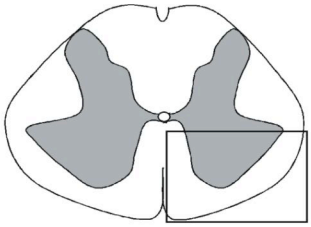
mice	treatmet	disease incidence		average day of onset ±SEM[a)]	mean maximal clinical severity ±SEM[a)]
wt	control	5/6	83%	12±1,0	2,7±0,5
wt	8-18C5	6/6	100%	10±2,4	3,13±1,0
C1q$^{-/-}$	control	5/5	100%	12,5±1,9	2,75±0,5
C1q$^{-/-}$	8-18C5	5/5	100%	12,4±2,1	2,7±0,7

[a)] diseased animals only

Figure 10 | *Demyelinating Abs drive the disease process in a complement dependent fashion.* wt (circles) and C1q$^{-/-}$ (triangles) mice were immunized with MOG_{35-55} in CFA and at days -1, 3, 5 and 9 of immunization animals were given 150µg of either α-MOG mAb (filled symbols) or equal concentration/volume of isotype control Ab or PBS (open symbols) by i.v. injection into the tail vein. Mice were examined daily for clinical disease and the table corresponds to the graph shown above. The figures are representative of at least two separate experiments (total n≥10) and were analyzed for statistical significance using a Student's t-test (*<0,05, #<0,01).

Histopathological analysis revealed that anti-MOG-mAb induced demyelination is completely dependent on the activity of complement. Macrophages are visible in areas of demyelination and constitute the majority of the infiltrated cells (Figure 12). In contrast to FcRγ$^{-/-}$ or wt mice, injection of anti-MOG-mAb in C1q$^{-/-}$ mice did not affect the degree of demyelination or inflammation (Figure 12). While the complement cascade appears to be critical for the demyelination mediated by the injection of anti-MOG-mAb, lack of complement per se did not alter the disease course evident by comparing untreated MOG-immunized C1q$^{-/-}$ and wt mice (Figure 10) emphasizing again that MOG-induced EAE in C57BL/6 mice develops completely independent of anti-MOG Ab raised by the immunization.

Figure 11 | *Histological analysis of CNS tissue from FcRγ$^{-/-}$, $J_H^{-/-}$ and $J_H^{-/-}$/FcRγ$^{-/-}$ mice.* Paraffin-embedded spinal cord lateral column white matter of lumbar sections were prepared from perfused wt, $J_H^{-/-}$, FcRγ$^{-/-}$ and $J_H^{-/-}$/FcRγ$^{-/-}$ animals at day 35 after induction of EAE with MOG_{35-55}. Hematoxylin and eosin (H&E) stains infiltrated cells, whereas luxol fast blue (LFB) shows myelin. Immunocytochemistry for $CD3^+$, MAC-3^+ and $B220^+$ subsets, shows infiltrated T cells, macrophages and B cells respectively. Scale bar, 200µm.



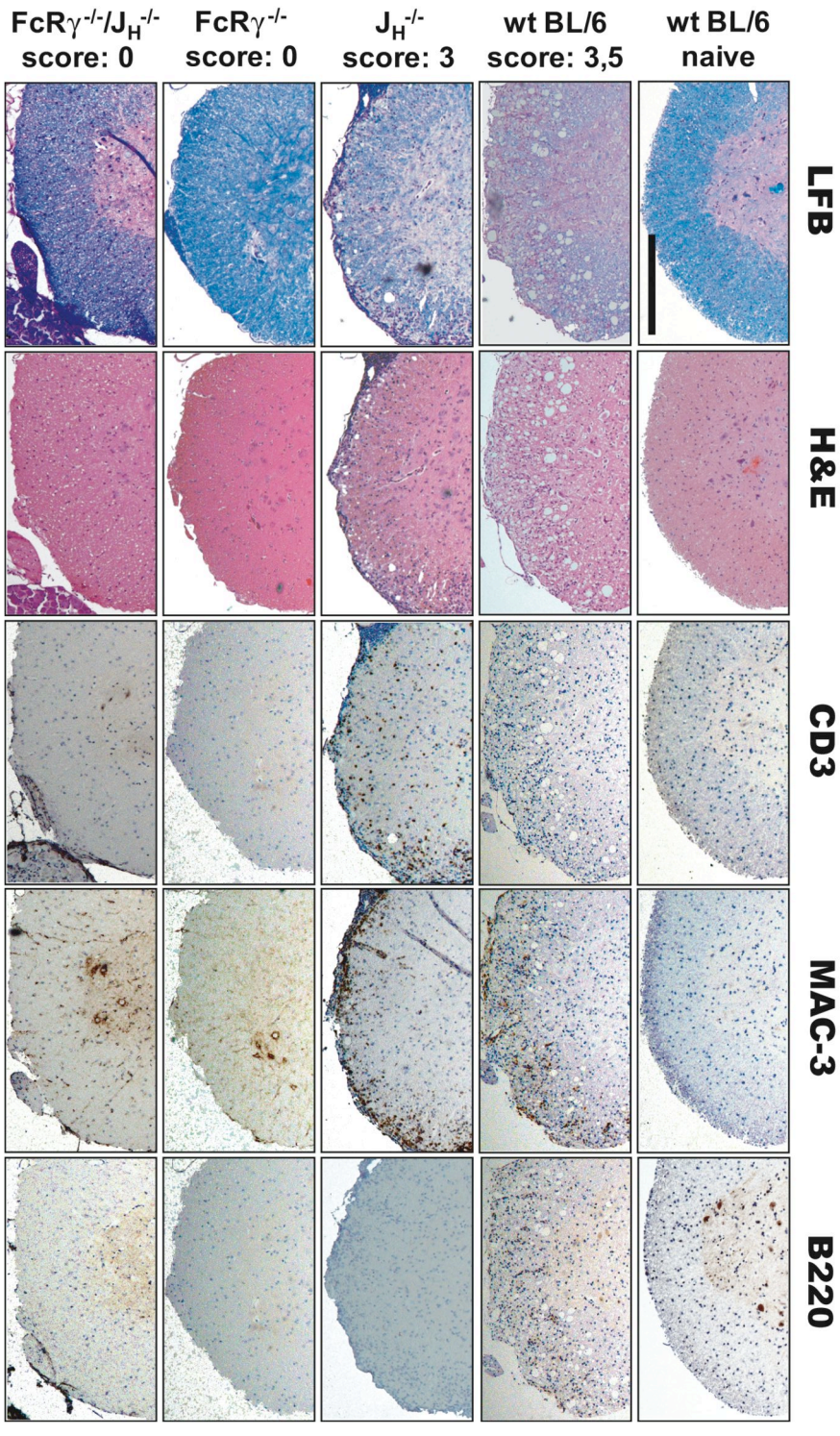
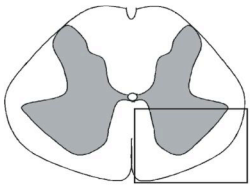
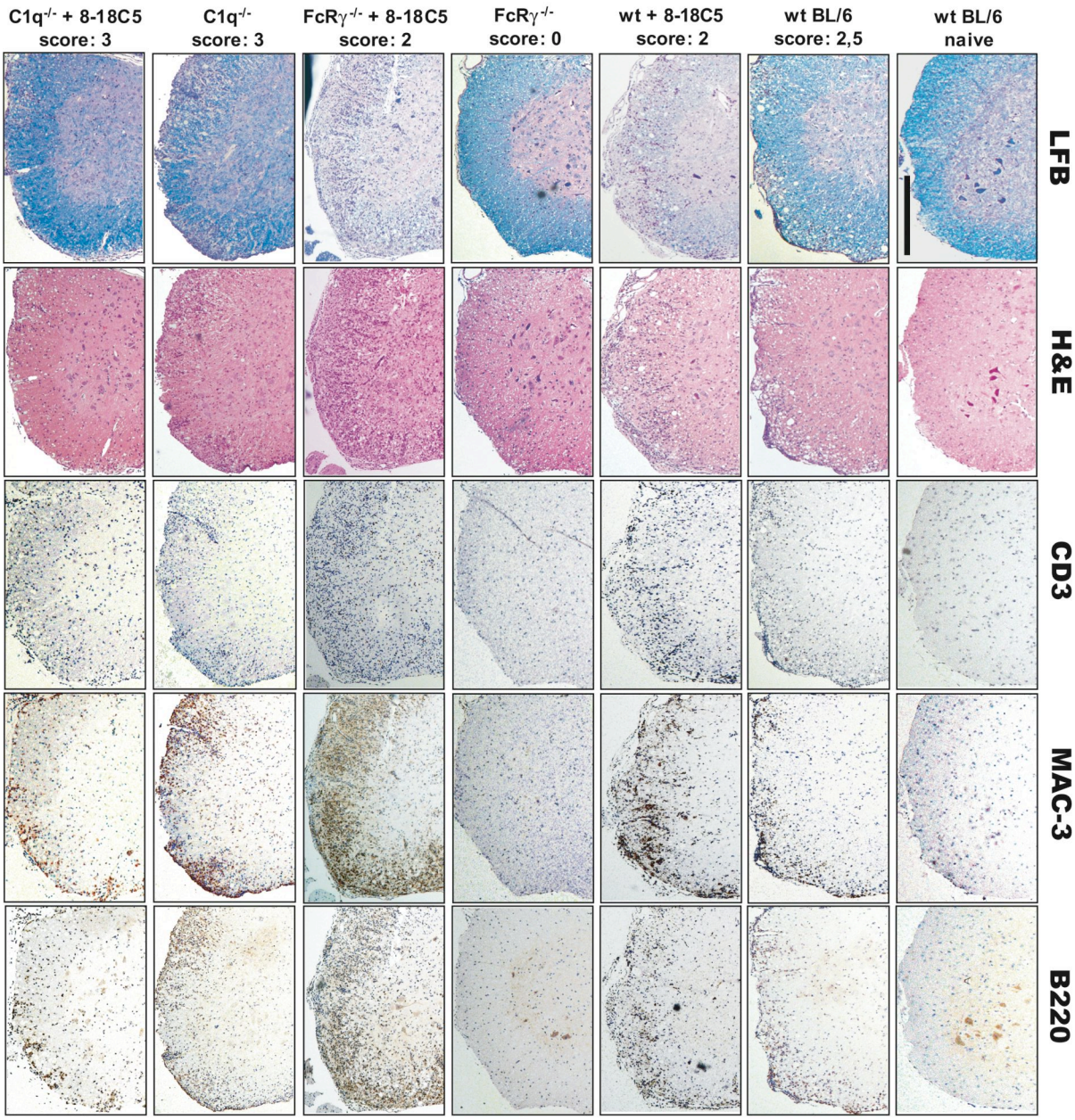


Figure 12 | *Mouse Histological analysis of CNS tissue from α-MOG mAb treated C1q$^{-/-}$ and FcRγ$^{-/-}$ mice.*
Spinal cords were isolated 19 dpi from randomly selected and perfused wt, FcRγ$^{-/-}$ and C1q$^{-/-}$ mice and embedded into paraffin. Representative lumbar sections were analyzed for demyelination and cell infiltration. Demyelination is depicted by luxol-fast blue staining (LFB), and cellular infiltration by hematoxylin and eosin (H&E). Infiltration of T cells (CD3), macrophages/monocytes (MAC-3) and B cells (B220) where also compared. Scale bar, 200µm.





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Chapter 4

Discussion

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1. Discussion of the role the role of B cells and Abs in the pathogenesis of EAE

Although B cells and plasma cells are known to be associated with MS lesions, information on their physiological and pathological function in the brain is still sparse. The histological findings suggest a role for B cells in the disease process. B cells and plasma cells are numerous in chronic lesions and in areas of active myelin breakdown[1-8], although it is not clear if CNS invading B cells are not an epiphenomenon as a results of antigen spreading. Elevated Ig levels are present in the CSF of more than 90% of patients with definite MS[7, 9]. Despite the apparent involvement of B cells in MS, data obtained in the EAE model have been conflicting. We wanted to assess their contribution to the disease process of EAE and sought to resolve the conflicting information surrounding the role of humoral immunity in EAE.

Here we used an array of different mutant strains and different genetic backgrounds and failed to establish any disease promoting function of B cells or Abs in MOG-peptide induced EAE. In contrast to the results we have obtained in our genetically B cell-deficient mouse model, previous studies in rat and murine models that report that EAE induction does not occur in B cell-depleted animals[10, 11]. Our results in MOG peptide-immunized BL/6 and $J_H^{-/-}$ mice are in agreement with others showing that MBP- or MOG-peptide immunized, B cell-deficient B10.PLμMT ($H\text{-}2^u$) and C57BL/6μMT ($H\text{-}2^b$) develop severe EAE[12-16]. It has been suggested that B cells, while not required for peptide-induced EAE development, are vital for processing and presenting full-length protein[12, 15, 17, 18]. However, two important differences exist between our B cell-deficient mice and those previously used.

First of all, the earlier B cell-deficient animals were obtained by depletion of B cells with infusion of anti-IgM Abs in neonatal animals[10, 11]. Later studies suggested that anti IgM-treated animals were defective in other aspects of the immune response, including T cell activity, making interpretation of the original studies difficult[19-21]. Secondly, the previous studies used μMT mice[12-16, 22, 23], which have a disrupted membrane μ-heavy chain[24]. However, μMT mice have been shown to harbor IgA and IgA-producing B cells casting doubt upon the applicability of this B-cell deficiency model[25]. Our mice ($J_H^{-/-}$), which have a targeted deletion of the heavy J gene segments, completely lack Ig expression and mature B cells[26]. Thus model has the advantage of eliminating any possibility of B cell leakage or unknown deleterious effects on other APC from infusion of exogenous protein.

In agreement with Lyons and colleagues, we demonstrate a requirement for B cells in EAE induction and progression depending on the nature of the Ag[15]. Our data suggest that B cells play a central role in the initiation of protein-induced EAE but not EAE induced by an encephalitogenic peptide from the same protein and that the lack of EAE in the $J_H^{-/-}$ mice was not due to the lack of T cell priming to MOG protein. Previous studies have suggested that different APC population are required for presenting peptide versus protein Ag to naïve T cells[27, 28]. DCs are most efficient at priming a peptide-induced response, whereas B cells were critical for the priming to some protein Ag[11, 29, 30]. Although in some others studies B cells appeared to be generally crucial for T cell priming in vivo[27, 31-39] or were able to stimulate T cells in vitro[40-51], our data exclude the possibility that B cells influence EAE solely through antigen presentation for T cell activation[27, 52, 53].

Our data support the hypothesis that the initiating APCs in a immune responses to peptide antigens are most likely DCs. DCs efficiently take up, process, and present soluble protein, and can stimulate naïve T cells more efficiently than B cells or macrophages[43, 54-61]. Together, the results described here indicate that MOG protein immunized $J_H^{-/-}$ mice could process and present Ag and were primed to the encephalitogenic MOG $_{35-55}$ epitope *in vivo*. It is possible that processing and presentation of other epitopes present in MOG protein could differ between wt and B cell$^{-/-}$ mice, and that these other epitopes may affect disease expression in B cell$^{-/-}$ mice. Furthermore, the interaction between T and B cells is thought to play a major role in determining to which epitopes generate an immune response i[35, 62-65]. Perhaps B cell$^{-/-}$ mice are incapable of inducing the T cell response, since they fail to present the dominant encephalitogenic epitope or to respond to other, uncharacterized EAE-enhancing epitopes within the MOG protein[66]. The MOG gene is located within the MHC, and low levels of MOG transcription are reported to occur within primary and secondary lymphoid organs[67-69]. This observation raised the possibility that strain specific differences in MOG expression outside the CNS could influence the composition of the B cell repertoire. An alternative mechanism could be that the presence of a conformational mimic of MOG encoded within the MHC could mediate a tolerogenic event[70]. However, in analogy to molecular mimicry in which structural and sequence homologies between foreign and self Ags can trigger autoimmunity and in some cases tolerance, it is possible that tolerance to one self Ag may be induced by cross-reactivity of the B cell receptor with another component of self[71-73].

Studies of the anti-MOG antibody repertoire[18, 70, 74-76] indirectly suggest that recognition of conformational determinants of MOG protein may be an important

requirement for pathogenicity. Showing that in terms of effector mechanisms, IgG deposition and complement activation occur exclusively in association with these conformational Abs[77]. It has been previously been established that the mAb 8-18C5 binds to discontinuous (i.e. conformation-dependent) determinants but not to linear MOG peptides[78, 79].

Von Buedingen et al. reporte that Abs raised in marmosets against rat MOG peptides could not transfer EAE to MBP primed animals, whereas antisera that include antibodies recognizing conformational determinants transferred a severe disease with regards to CNS lesions and demyelination[77]. We however were able to dismiss the possibility of a crucial role for autoreactive conformational Abs in trafficking encephalitognic T cells into the CNS or a role for Abs immune regulation in MOG protein induced EAE. Also, our findings concerning the Ab-independent contribution of B cells altering T cell activation and expansion are in concordance with published data, which showed that efficient *in vivo* priming of T cells, to both peptides and proteins, occurs in B cell-deficient mice[52]. It is possible that internalization of Ag through the BCR could generate a different set of epitopes that are presented to T cells[27, 80-82], thereby activating a unique subset of Ag-specific T cells. Studies of auto-Ag have shown that B cells elicited with foreign cross-reactivity Ag can present self-peptides, resulting in priming of autoreactive T cells[66, 83].

Mice genetically deficient in mature B cells and immunized with MBP resulted in induction of EAE with impaired recovery when compared to wt mice[12]. B cell-deficient mice failed to recover from acute EAE, suggesting a role for B cells in immune regulation. In addition, recent studies indicate that regulatory B cells develop in

several murine models of chronic inflammation, including rheumatoid arthritis[84, 85], lupus[86], bowel disease[87-89], autoimmune diabetes[90] and EAE[12, 14, 91, 92]. Fillatreau et al. demonstrate that recovery from EAE was dependent on the presence of autoantigen-reactive B cells[14]. The regulatory function may be directly accomplished by the production of regulatory cytokines IL-10 and TGF-β or by the ability of B cells to interact with pathogenic T cells to dampen harmful immune responses[93]. In EAE, both B cells and regulatory T cells are involved in the regulation of CNS autoimmune disease; B cells may limit the continued expansion of fresh pathogenic T cells from lymph nodes, whereas regulatory T cells directly control the disease at the site of inflammation[94] or they suppress inflammation by restoring the Th1/Th2 balance[14] through skewing of Th cells to a Th2 response. An absence of Th2 cytokines may allow continued expansion and migration of activated Th1 encephalitogenic cells into the CNS, thus prolonging inflammation and demyelination in the target organs. Activation of T cells also needs an “unspecific” costimultory support in addition to the “specific” TCR-mediated signal between APC and T cell. B cell may be an important cellular factor in supporting T cell activation in the immune periphery and the CNS.

2. Discussion of the contribution of FcR and complement to EAE

Ab-mediated tissue damage is widely held to be mediated primarily through the action of immune complexes (IC), complement- and FcR activation. The FcRγ chain is in this context not only important for cell activation but also necessary for the efficient assembly and cell-surface expression of these four types of FcRs, including FcRI, FcγRIII, FcεRI and FcαRI. Therefore, FcRγ deletion results in the combined deficiency of activating type FcRs[95]. FcRs have been implicated in the pathogenesis

of EAE due to the fact that FcRγ$^{-/-}$ mice are relatively EAE-resistant even though they develope comparable levels of MOG-specific Abs[96, 97]. Our findings are in agreement with earlier studies, which also reported drastically attenuated EAE in FcRγ$^{-/-}$ on a DBA/1 and B6-129-background[22, 96-98].

Several independent studies support the notion that CNS-resident cells, in particular microglia, recognize IC and Ab-deposits within the CNS leading to phagocytosis of opsonized target cells[99]. However, deletion of FcRγ from the CNS-parenchyma did not impact on EAE susceptibility, clinical disease or demyelination, whereas the absence of FcRγ in the peripheral immune compartment conveys protection. Accordingly, the resistance of FcRγ$^{-/-}$ mice to developing EAE is not due to the inability of CNS-resident cells to recognize and become activated by Abs or ICs.

A vital role for Ig-binding FcRs is in apparent conflict with our failure to establish any pathogenic function of B cells in EAE: To resolve this paradox, we demonstrated that the deficiency in FcRγ lesions immunity independently of B cells or Igs and that the EAE-resistance of FcRγ$^{-/-}$ mice cannot be attributed to humoral immunity. The notion that FcRγ functions independent of FcR mediated Ab-recognition is supported by the recent report by Szalai et al., which demonstrates that the Ab-binding FcRα units are not required to develop EAE[100]. FcRγ is not only critical for FcR-mediated signaling, but has also been shown to be involved in the signal transduction of several other FcR-unrelated receptor complexes on different cell types: NKR-P1 on NK-, NK T- and dendritic-cells (DCs); αβ-TCR on T cells; Pir-A on DCs, macrophages and B cells and GPIb on platelets. Furthermore FcRγ is closely related to the ζ-chain of the

T cell antigen receptor (TCR)/CD3 complex and in fact FcRγ-ζ heterodimers and FcRγ-FcRγ homodimers can participate in TCR formation and function[100-105]. Szalai et al. suggested that FcRγ signaling in γ/δ T cells could explain the EAE resistance of FcRγ$^{-/-}$ mice[100].

They implied that expression of FcRγ by γδ T cells, probably in conjunction with the T cell receptor/CD3 complex, is potentially required for full development of EAE[100]. Our data however, dismiss any vital FcRγ function in B or T lymphocytes and point towards a crucial role of FcRγ in the accessory cell compartment. Reconstitution of FcRγ-deficient mice with wt accessory cells renders them fully EAE susceptible, even when lymphocytes remain FcRγ-deficient. We suspected that the loss of FcRγ impairs APC capacity and thus the expansion of encephalitogenic T cells. However, our data dismiss the involvement of FcRγ in APC function or T cell expansion. In this context, we found that FcRγ does not affect the induction of DC maturation, Ag-processing or presentation. In support of these findings, FcRγ$^{-/-}$ mice do not develop EAE even when an expanded population of fully primed encephalitogenic lymphocytes is adoptively transferred, again underlining that FcRγ-mediated accessory cell activation is required for the effector phase of EAE.

Murine models of autoimmune disease indicate the indispensable roles of the inhibitory FcRs in the suppression of such disorders, whereas activating-type FcRs are crucial for the onset and exacerbation of the disease. The development of many autoimmune diseases may be caused by impairment of the Fc receptor regulatory system[95]. It is feasible that the EAE resistance of FcRγ$^{-/-}$ mice is caused by

unbalanced steady state signal transduction, skewing the response towards attenuated status. This notion is supported by studies with mice lacking another ITAM-bearing adapter molecule, namely DAP-12, which were also resistant to MOG-peptide induced EAE[106].

Active immunization with either MOG-protein or peptide leads to the production of αMOG specific Igs *in vivo*. While we cannot demonstrate any pathogenic function of these Abs, inoculation with specific αMOG Abs *in vivo* drastically exacerbates clinical disease and demyelination. Also, Litzenburger et al. found that transgenic mice expressing a B cell receptor specific for MOG, while not developing spontaneous disease, develop exacerbated EAE after immunization[79]. In line with the idea that the impact of the FcRγ in EAE is B cell and Ab-independent, FcRγ deficient mice are rendered susceptible to EAE when inoculated with demyelinating αMOG Abs. Interestingly, while succumbing to clinical EAE after αMOG Ab injection, FcRγ$^{-/-}$ mice still displayed a significant delay in disease onset supporting that the FcRγ function in EAE is unrelated to Abs. The fact that complement-deficient mice are fully EAE susceptible again supports the notion that humoral immunity is not relevant for EAE development induced by active immunization. However we demonstrate that large amounts of inoculated pathogenic Abs mediate their disease promoting function exclusively via the complement cascade. The role of complement activation in EAE has been debated[107-109]. Loss of C3 attenuates EAE[110] and expression of the complement inhibitor sCrry prevents EAE[111]. Also, complement activation appears to be crucial in the context of EAE exacerbated by the inoculation of demyelinating Abs[112-115]. On the other hand, Boos et al.[108] and Calida et al.[109] could demonstrate that key components of the complement system, such as C3 and C4, are not

involved in MOG_{35-55} induced EAE pathogenesis. We found that complement activation mediated by C1q is crucial for the demyelinating effects of α-MOG-Abs, yet that loss of this pathway does not impact on disease development in the absence of inoculated demyelinating Abs.

Taken together, our findings help explaining the discrepancies and controversial findings with regards to humoral immunity in EAE. In MS, enhanced titers of anti-myelin antibodies in serum and CSF have been described in MS patients and in a subgroup of patients with clinical isolated syndrome the levels of anti-MOG Abs have been suggested to be useful as a prognostic marker[3]. In EAE, we did not observe any correlation between Ab-titers and EAE severity. In fact, it is feasible that the B cell activity and the intrathecal presence of B cells serve a regulatory attenuating function in MS[14, 92, 93]. The presence of Ig and complement deposits suggests that anti-myelin Abs are involved in the demyelinating process. The fact that administration of αMOG Abs exacerbates EAE is in agreement with the conclusions drawn from the histopathological analysis of MS tissue. In particular, Lucchinetti and coworkers identified a subtype of MS pathology in which elevated levels of Ab, complement and plasma cells can be observed in situ[2, 116]. Modeling this pathology, administered αMyelin Abs can clearly exacerbate demyelination and clinical disease. However, a pathogenic function of host-derived Abs raised by the active immunization cannot be established in the standard models of EAE.

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Chapter 5

Material and Methods

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1. Mice

C57BL/6-CD45.1 and congenic C57BL/6-CD45.2 mice were obtained from Harlan Laboratories (Netherlands), FcRγ$^{-/-}$ and $J_H^{-/-}$ mice from Taconic (Germantown, NY). MOG-specific TCR transgenic (Tg) C57BL/6 (2D2) mice were provided by V.J. Kuchroo[1] (Harvard Medical School, Boston, MA). C1q$^{-/-}$ mice[2] were obtained from A. Aguzzi (University Hospital, Zurich) and the SMARTA p11 TCR-Tg and Rag1$^{-/-}$ were provided by R. Zinkernagel (University Hospital, Zurich)[3]. μMT mice were kindly provided by Dr. Charles A. Janeway Jr. All mice were bred at the animal facilities of the University of Zurich under specific pathogen-free conditions. Animal experiments and breeding were approved by the Swiss Veterinary Office (#69/2003, #70/2003 and #13/2006).

2. Reagents

Murine His_6-tagged recombinant mouse (amino acids 1-121)[4] and human (amino acids 1-125) MOG[5] was expressed in *E.coli* and purified using metal chelate chromatograph. MOG_{35-55}-peptide (MEVGWYRSPFSR VVHLYRNGK) and SMARTA (LCMV) p11 (GPDIYKGVYQFKSVEFD) were synthesized by GenScript, (Piscataway, NJ). Keyhole limpet hemocyanin (KLH) was purchased from Fluka (Buchs, CH), Concanavalin A and LPS were bought from Sigma (Switzerland), mouse monoclonal anti-MOG antibody 8-18C5 (IgG_1)[6] was provided by Prof. N. Goebels (University Hospital, Zurich).

3. Bone marrow chimeric mice

Irradiation Bone-marrow (BM) chimeric mice were generated as described previously[7]. BM donor mice were killed using CO_2 and BM cells were isolated by flushing femur, tibia and hip bones with PBS. Bone marrow cells were then passed through a cell strainer with pore size of 100μm and cells were washed with PBS. Recipient mice were lethally irradiated with 1100 rads (split dose) and were intravenously injected with $20x10^6$ donor BM cells. Engraftment took place over 8 weeks of recovery, before the mice were used in EAE experiments.

4. Induction of EAE and clinical assessment

EAE was induced by s.c. flank injection of 200µg of MOG_{35-55}-peptide or rMOG-protein emulsified (1:1) in Complete Freund's Adjuvant (CFA) (Difco, Detroit, MI) on day 0, supplemented by i.p. injections of 200ng of pertussis toxin (PT) (Sigma) on day 0 and 2.

For adoptive transfer studies, donor mice were immunized as described above. 11 days later the mice were sacrificed, spleen and LNs were removed, homogenized and red blood cells lysed. For lysis of red blood cells, spleenocytes were incubated on ice for 10 minutes in lysing buffer (containg NH_4CL, $KHCO_3$ and EDTA). Cells were filtered through a 100µm cell strainer and were subsequently cultured for 4 days in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin and in the presence of 50µg/ml MOG peptide and 2,5ng/ml IL-12 at a concentration of $4x10^6$ cells/ml. MOG_{35-55}-reactive lymphocytes were then washed and injected into recipient mice (20-30 x 10^6 cells/mouse). Animals also received 200ng/mouse PT several hours later and on day 2 post-cell transfer.

The mice were evaluated daily for clinical signs of disease on a scale of 0-5 with gradations of 0.25 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, limp tail and hind limb weakness (waddling gait); 3, hind limb paralysis; 4, hind and fore limb paralysis (score > 4 to be sacrificed); 5, death. Each time point shown is the average disease score of each group. Statistical significance was assessed using an unpaired Student t-Test.

5. LTβR-Fc treatment

C57BL/6 mice were injected i.p. with 100 μg of human LTβR-Fc Fusion protein (generously provided by Dr. Yang Xin Fu, University of Chicago) or 100 μg human IgG control (Bioexpress) 7 days prior to immunization with MOG and weekly thereafter.

6. Generation of bone marrow-derived DCs

Femurs from bone marrow donor mice were removed and bone marrow cells were isolated by flushing of the bones with PBS; cells were then filtered through a cell strainer with a pore size of 100 μm. Cells ($2x10^5$ cells/ml) were cultured in RPMI medium containing 10% FCS with the addition of 10% conditioned medium obtained from X-63 cells transfected with a plasmid containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (obtained from A. Rolink, University of Basel, Switzerland). After at least 6 days, bone marrow–derived DCs were matured with 10 μg/ml of lipopolysaccharide overnight; immature bone marrow–derived DCs were maintained in medium containing GM-CSF. Bone marrow–derived DCs were used from day 7 to day 9.

7. T cell expansion assays

Mice were immunized by flank injections of CFA alone, MOG_{35-55}/CFA or MOG_{1-121}/CFA. After 7 days, the axillary and inguinal lymph nodes (LN) as well as spleens were removed and homogenized. $2x10^5$ cells were seeded as triplicates in a 96 well plate and pulsed with 0.5, 5 and 50µg/ml MOG or 5µg/ml concanavalin-A (Fluka, Buchs CH). After 24 hours, 1µCi ^{3}[H] thymidine (Fluka, Buchs CH) was added and incubated for an additional 24 hours before cells were harvested using a Filtermate harvester (Inotech, Dottikon CH) and thymidine incorporation was assessed and Wallac MicroBeta® TriLux- scintillation-counter (PerkinElmer). For cytokine analysis, sister cultures were harvested 48 hours after culture and supernatants were analysed by ELISA for IFN-γ and IL-2 (BD PharMingen).

For in *vitro* proliferation of transgenic T cells, spleens were collected from naive transgenic mice and $CD4^+$ T cells were purified with BD-IMag magnetic beads (BD Pharmingen). The purity of T cell isolation was verified by flow cytometry. The transgenic T cells ($1x10^5$) were cultured in a 96-well plate together with immature or mature bone marrow DCs. Before coculture, bone marrow DCs were pulsed for 3h with 1 mg/ml of Tg TCR specific peptide in RPMI medium, then were washed and were irradiated with 2,000 rads. Unpulsed DCs were used as a control, as were T cells cultured alone. Cells were incubated for 2 days and [3H]thymidine was added for the last 18 h of culture.

For *in vivo* proliferation assays, mice were immunized with 200 µg MOG_{35-55}, $rMOG_{1-121}$ emulsified in CFA or CFA alone. Several hours later, the mice were injected i.v. with CFSE (carbofluorescein diacetate succinimidyl ester)-labeled splenocytes (10µM) (Invitrogen - Moleculare Probes) obtained from TcR Tg mice. 4 days later, spleens and LNs are isolated from immunized mice and proliferation of transgenic CD4 T cells was performed by FACS analysis using specific antibodies to TCR Vα3.2 and Vβ1.1.

8. Enzyme-linked immunosorbent assay (ELISA)

The culture supernatants (described above) were analyzed using commercially available murine ELISA-kits for IFN-γ and IL-2 (BD Biosciences Mountain View, CA), according to the manufacturer`s instructions. For determining Ig-isotype ELISA plates were coated with 10µg mouse $rMOG_{1-121}$ in 0.1 M $NaHCO_3$ (pH 9.6) over night (o.N.) at 4°C and blocked with 1% (w/v) bovine serum albumin (BSA). Serial dilutions of serum in PBS were added to Ag-coated wells and incubated o.N. at 4°C. After washing, affinity-purified peroxidase-conjugated goat Abs to mouse Igs, IgG or IgM (Sigma, St. Louis, MO) were added, at a dilution of 1:1000 in PBS. Binding was revealed using stabilized chromogen (Biosource, Belgium) and the absorbance at 450nm was read on an micro plate reader (Bio-Rad, CA USA).

9. Enzyme Linked Immuno Spot Technique (ELISpot)

For ELISpot assays, lymphocytes were isolated from LNs and spleen of MOG_{35-55}- and MOG_{1-121}-immunized mice on day 7 dpi, as described above. Cells (2×10^5) were plated per well in complete RPMI medium containing 50 µg/ml MOG_{35-55} in 96-well plates (Millipore) coated with 7.5 µg/ml anti-IFN-γ (AN18; Mabtech) or 2

µg/ml anti-IL-17 (TC11-18H10; BD Pharmingen) Abs. The plates were incubated at 37°C, 5% CO_2 for 18 (anti-IL-17) or 20 (anti-IFN-γ) hours at which point cells were discarded and plates were washed with PBS. Then, 0.5 µg/ml of biotin-conjugated anti-IFN-γ (R4-6A2; Mabtech) or 1 µg/ml of biotin-conjugated anti-IL-17 (TC11-8H4.1; BD Pharmingen) detection Abs was added and incubated at 25°C for 2h and 4 h, respectively. After plates were washed, streptavidin-alkaline phosphatase (Mabtech) was added, followed by incubation for 1 h at 25°C. Plates were washed with PBS and 75 µl of the substrate solution BCIP/NBT-plus (5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium) was added to the wells, which were developed until distinct spots emerged. Plates were analysed with an enzyme-linked immunospot reader (Immunospot; CTL).

10. Histology

Mice were transcardially perfused with 20ml PBS and subsequent with 4% (weight/volume) paraformaldehyde in PBS. Spinal columns were removed and were fixed in 4% (weight/volume) paraformaldehyde in PBS. Spinal cords were then dissected and were embedded in paraffin before being stained with either hematoxylin and eosin or with anti-CD3, anti-B220 or anti-Mac-3 antibodies (BD Pharmingen and Serotec) to assess infiltration of inflammatory cells. Luxol fast blue stain was used to determine the degree of demyelination .

11. Flow cytometry

For Flowcytofluorometric analysis we used the following Abs diluted in FACS buffer (PBS supplemented with FCS and sodium azide): anti-CD45 (30/F11), anti-CD4 (RM4-5), anti-Vα3.2 (RR3-16), anti-CD11b (M1/70), anti-CD11c(GS-32), anti-GR-1

(RB6-8C5) and anti-B220 (RA3-6B2), anti-CD40 (3/23), anti-CD62L (Mel-14), anti-CD162 (2Ph1), anti-VLA-4 (R1-2), anti-CD5 (53-7.3), anti-CD25 (PC61), anti-Ly5.1 (104), anti-Ly5.2 (A20). All antibodies were purchased from BD Pharmingen. For analysis of CNS invading cells, mice were euthanised with CO_2 and perfused intracardially with PBS. Spinal cord was flushed out with PBS and the brain was dissected to isolate the brainstem. Tissues were digested using liberase treatment (400 µg/ml liberase (Roche) and 200 µg/ml DNase (Roche)) at 37°C for 30 min. The tissues were then homogenised and strained through a 100µm nylon filter (Fisher). After centrifugation, cell suspensions were resuspended in 30% Percoll (Pharmacia) and centrifuged at 18,500g for 30 min at 4°C. Interphase cells were collected and washed extensively before being staining. For flow cytometry, we incubated the primary antibody for 20 min at 4°C. Cells were then washed with FACS buffer and centrifuged for 2 min at room temperature (RT) prior to staining for 15 min with the secondary antibody at 4°C. Cells were washed, centrifuged and resuspended in 300 µl facs buffer for subsequent analysis. We analysed the cells using a FACS-Canto (BD Pharmingen) with Cell-Diva™ software. Post-acquisition analysis was performed using FLOWJO™ software.

12. Protein purification

The expression of recombinant MOG was performed in transformed E. coli bacteria of the DH5α strain. The expression vector introduced contains an IPTG inducible promoter, the cDNA coding for the extracellular domain of mouse and human MOG and a histidine tag sequence coding for 6 histidines, to allow purification by nickel-chelate chromatography. 600ml of selective culture medium was inoculated with an transformed E. coli glycerol stock and incubated overnight at 37°C. For 30 minutes

the bacterial pellet was then resuspended in 6 x 1 liter of selective culture medium (LB medium, 50 µg/ml ampicillin) and shaken at 37°C. IPTG (BioTech Trade) were added per liter of culture to induce MOG expression. Incubation was continued for a further 3-4 hours, bacteria were centrifuged (20min, 5000rpm, 4°C) resuspended in 200ml PBS and centrifuged again. The pellet was stored overnight at -20°C. The pooled bacterial pellet was resuspended in 40ml PBS and sonicated for 10 minutes on ice (Bioblock Scientific, France) to lyse the cells. Recombinant MOG is insoluble in PBS and forms inclusion bodies. After centrifugation (120min, 12000rpm, 4°C) containing the MOG inclusion bodies and other insoluble proteins was resuspended in urea buffer (6M urea; 0.5M NaCl; 0.1M KH2PO4; pH 8) and sonicated for 10 minutes to dissolve the inclusion bodies. The suspension was again centrifuged and the supernatant containing the dissolved MOG was used for affinity chromatography.

The affinity column (HiTrap Chelating HP 5ml, Amersham) was washed sequentially with dH2O and with 1% EDTA/0.05% Tween20 to remove the 70% ethanol used for storage and any remaining nickel. The column was loaded with a 1% nickel (Ni 2+) solution, washed with dH_2O and equilibrated with urea buffer. The sample was loaded on to the column, and the flow rate was set (1ml/min). The column washed with 50ml wash buffer (40 mM imidazole in urea buffer). The elution was performed with an linear imidazole gradient with 50ml elution buffer (500 mM imidazole in urea buffer). Fractions of 2ml were collected and analysed by SDS-PAGE. The protein concentration in the eluate was monitored by photometer at 280nm. After SDS-PAGE analysis the protein-containing eluate fractions were pooled and placed in a dialysis cassette (Pierce, Switzerland) in 5 liters PBS. Dialysis was performed twice overnight at 4°C against PBs and the MOG preparation was verified by SDS-PAGE.

13. SDS-Page

SDS-Page was carried out according to the method described by Laemmli[8] using 12% polyacrylamide gels. Transfer of protein from gel to blots was done using the wet method. Nitrocellulose membranes, stacking filter paper and resolved gel were soaked in transfer buffer and stacked on top of each other such that membrane, gel and filters were aligned exactly on top of each with gel on cathode side and the electrical leads connected to a power supply. A current of 0.65mA/cm2 gel was applied for a period of 1 hour. To reveal separated proteins on the nitrocellulose membrane, membrane was stained with 0,2% Ponceau-staining solution (Biorad, Germany) for 10 min and destained with dH_2O. After blotting, membranes were blocked for 1 hour at room temperature in blocking buffer (1% FCS in PBS). Membranes were incubated with unlabeled specific primary monoclonal antibody over night at 4°C. Following a series of washing steps, membranes were incubated for 1h with a secondary antibody coupled to horse radish peroxidase. Specific bands were detected using the ECL-pico system (Pierce, Switzerland). The membranes were exposed to film in the dark for a time period, ranging from a few seconds to 1h and developed using a Kodak X-OMAT film-developing machine.

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- Biochemistry (University of Konstanz)
- Molecular Toxicology (University of Konstanz)
- Biochemical Pharmacology (University of Konstanz)

PUBLICATIONS

Articles:

E. Urich, L.M. Howard, S.D. Miller, A. Waisman and B. Becher
Importance of B cells as APCs in neurodegenerative diseases
(in preparation)

S. Kuenzle, H.C. von Buedingen, M. Meier, M.D. Harrer, **E. Urich**, B. Becher and N. Goebels
Pathogen specificity and autoimmunity are distinct features of antigen driven immune response in neuroborreliosis
(submitted)

E.Urich, I. Gutcher, M. Prinz and B. Becher
Autoantibody-mediated demyelination depends on complement activation but not activatory Fc-receptors
Proc. Natl. Acad. Sci. USA 103, 18697-18702 (2006)

I. Gutcher, **E. Urich**, K. Wolter, M. Prinz and B. Becher
Interleukin 18-independent engagement of interleukin 18 receptor-α is required for autoimmune inflammation
Nat. Immunology 7, 946-953 (2006)

M. Polymenidou, F. Heppner, C. Pellicioli, **E. Urich**, G. Miele, B. Becher and A. Aguzzi
Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection
Proc. Nat. Acad. Sci. USA 101, 14670-14676 (2004)

Reviews:

E. Urich and B. Becher
Role of B cells and humoral Immunity in Multiple Sclerosis
Leading Opinions in Neurology and Psychatry 5, 30-32 (2005)

Abstracts:

E. Urich, M. Prinz and B. Becher
Deletion of Fc-receptor-gamma abrogates encephalitogenicity of effector T cells in EAE
Journal of Neuroimmunology 154, 1-2 (2004)

ATTENDED MEETINGS

- 7.th International Congress of Neuroimmunology Venice, Italy 2004 (oral presentation)
- 6.th Neuroimmunology State of the Art Symposium Luzern, Switzerland 2004
- NCCR Neuroscience Symposium Konstanz, Germany 2004 (poster presentation)
- Experimental Neuroimmunology Meeting Zurich, Switzerland 2005 (poster presentation)
- Neuroimmunology Symposium Seon, Germany 2004 and 2006 (oral presentation)
- MS Symposium Zurich, Switzerland 2004, 2005 and 2006
- Congress of Swiss Society for Allergology and Immunology Zurich, 2006 (poster presentation)
- XVIII. Meeting of Swiss Immunology PhD Students, Switzerland 2006 (oral presentation)
-NCCR Neuroscience Symposium Bern, Switzerland 2007 (poster presentation)
- 8th C.R. Brupbacher Symposium for Cancer Immunosurveillance & Immunotherapie

ABSTRACTS

E.Urich, I. Gutcher, M. Prinz and B. Becher
Autoantibody-mediated demyelination depends on complement activation but not activatory Fc receptors Proc.Natl. Acad. Sci. USA 103, 18697-18702 (2006)

The precise mechanisms leading to CNS inflammation and myelin destruction in both Multiple Sclerosis (MS) as well as Experimental Autoimmune Encephalomyelitis (EAE) remain subject of intense debate. In both MS and EAE, auto-antibodies (Abs) are thought to be involved in tissue destruction through recruiting Fc receptor (FcR)-bearing cells or direct cytotoxic effects through the activation of the complement pathway. While intrathecal immunoglobulin (Ig) production and Ig-deposition in inflammatory lesions is a hallmark of MS, mice deficient in B cells and Igs develop severe EAE. Paradoxically, mice of the same genetic background, but deficient in FcRγ, are EAE-resistant. We found that the functional expression of FcRγ on systemic accessory cells, but not CNS-resident cells, appears to be vital for the development of CNS-inflammation, independent of antigen presenting cell (APC)-function or antibody involvement. On the other hand, we found that the injection of anti-myelin oligodendrocyte glycoprotein (MOG)-Abs drastically worsens disease severity, inflammation and demyelination. Using $FcR\gamma^{-/-}$ as well as $C1q^{-/-}$ mice we could definitively establish that the demyelinating capacity of such auto-Abs in vivo relies entirely on complement activation and is FcR-independent.

I. Gutcher, E. Urich, K. Wolter, M. Prinz and B. Becher
Interleukin 18-independent engagement of interleukin 18 receptor-α is required for autoimmune inflammation Nature Immunology 7, 946-953 (2006)

T helper type 1 (TH1) lymphocytes are considered to be the main pathogenic cell type responsible for organ-specific autoimmune inflammation. As interleukin 18 (IL-18) is a cofactor with IL-12 in promoting TH1 cell development, we examined the function of IL-18 and its receptor, IL-18R, in autoimmune central nervous system inflammation. Similar to IL-12-deficient mice, IL-18-deficient mice were susceptible to experimental autoimmune encephalomyelitis. In contrast, IL-18Rα-deficient mice were resistant to experimental autoimmune encephalomyelitis, indicating involvement of an IL-18Rα ligand other than IL-18 with encephalitogenic properties. Moreover, engagement of IL-18Rα on antigen presenting cells was required for the generation of pathogenic IL-17-producing T helper cells. Thus, IL-18 and TH1 cells are dispensable, whereas IL-18Rα and IL-17-producing T helper cells are required, for autoimmune central nervous system inflammation.

M. Polymenidou, F. Heppner, C. Pellicioli, E. Urich, G. Miele, B. Becher and A. Aguzzi
Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection Proc. Nat. Acad. Sci. USA 101, 14670-14676 (2004)

Prion diseases are characterized by the deposition of an abnormal form (termed PrPSc) of the cellular prion protein (PrPC). Because antibodies to PrPC can antagonize deposition of PrPSc in cultured cells and mice, they may be useful for anti-prion therapy. However, induction of protective anti-prion immune responses in WTanimals may be hindered by host tolerance. Here, we studied the cellular and molecular basis of tolerance to PrPC. Immunization of *Prnp*o/o mice with bacterially expressed PrP (PrPREC) resulted in vigorous humoral immune responses to PrPREC and native cell-surface PrPC. Instead, WT mice yielded antibodies that failed to recognize native PrPC despite immunoreactivity with PrPREC, even after immunization with PrP-PrP polyprotein and or upon administration of anti- OX40 antibodies. Consequently, immunized WT mice experienced insignificantly delayed prion pathogenesis upon peripheral prion challenge. Anti-PrP immune responses in *Prnp*o/o mice were completely abrogated by transgenic expression of PrPC in B cells, T cells, neurons, or hepatocytes, but only moderately reduced by expression in myelinating cells, despite additional thymic *Prnp* transcription in each case. We conclude that tolerance to PrPC can coexist with immunoreactivity to PrPREC and does not depend on thymic PrPC expression. Its circumvention might represent an important step toward the development of effective anti-prion immunotherapy.

S. Kuenzle, H.C. von Buedingen, M. Meier, M.D. Harrer, E. Urich, B. Becher and N. Goebels
Pathogen specificity and autoimmunity are distinct features of antigen driven immune response in neuroborreliosis (submitted)

Neuroborreliosis (NB) is a chronic infectious disease of the central nervous system caused by a tick borne spirochaete, *Borrelia burgdorferii* (Bb). In addition to direct effects of the causative agent, additional immune mediated mechanisms are thought to play a role in the central nervous system (CNS) pathology of NB. In order to further understand the involvement of humoral immune mechanisms in NB, we dissected the intrathecal antibody responses down to the single plasma cell level. Starting with single cell RT-PCR of FACS sorted cerebrospinal fluid (CSF) plasma cells from a NB patient, we identified expanded clones and resurrected the antigen specificity of their secreted antibodies through recombinant expression of the correctly paired Ig heavy and light chain genes as monoclonal antibodies. As expected, we found specificity for the causative infectious agent Bb among the clonally expanded plasma cell (cePC) derived monoclonal antibodies. However, from an independent cePC of the same patient, we could derive mAbs specific for human CNS myelin, without detectable crossreactivity with Bb antigens. While reactivity against Bb is an known feature of humoral immune responses in NB, we are the first to show a) that immune responses specific for self antigens are a distinct feature of CNS infections independent of pathogen reactivity and b) that humoral autoimmunity in NB – since found in clonally expanded plasma cells - is the result of a truly antigen driven immune response. Our findings indicate, that in NB mechanisms are at play that induce distinct immune responses specific for pathogen and self antigens independent from molecular mimicry.